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STUDIES ON THE ACTIVE SITE AND SUBUNIT STRUCTURE OF
SUCCINYL-COENZYME A SYNTHETASE



by

MAUREEN O'CONNOR


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ABSTRACT

The keystone for understanding the catalytic and regulatory properties of many enzymes has been the recognition of their oligomeric status. Succinyl-CoA synthetase is known to be an oligomeric enzyme with a heterologous subunit structure; the enzyme from *E. coli* and other Gram-negative bacteria has a tetrameric $\alpha_2\beta_2$ structure *in vitro* while Gram-positive bacteria and diverse eukaryotic organisms produce a dimer of the $\alpha\beta$ type. *E. coli* and pig heart succinyl-CoA synthetase are studied in this thesis in an attempt to rationalize their complex subunit structures.

Affinity chromatography and modification studies on the *E. coli* enzyme were consistent with the previously conceived idea that the active site of the enzyme is assembled at the contact point of the two subunit types. The CoA and succinate binding sites appear to be located on the β subunit while the α subunit binds ATP and initiates phosphoryl transfer.

The negative cooperativity of phosphorylation exhibited by *E. coli* succinyl-CoA synthetase together with its ATP-dependent oxygen exchange pattern have been interpreted as reflecting the operation of catalytic cooperativity between active sites. The oxygen exchange kinetics of hybrid *E. coli* enzyme preparations were examined in an attempt to validate this hypothesis. These experiments were complicated

by the finding that the amount of oxygen exchange catalyzed by succinyl-CoA synthetase is a function of the concentration of enzyme used in the assay.

When this effect was investigated further for both the pig heart and *E. coli* enzymes it became apparent that the oxygen exchange kinetics could be adequately explained by a model not involving catalytic cooperativity but proposing a dimer-tetramer equilibrium for succinyl-CoA synthetase. An analysis of the partition coefficient of the exchange reaction under different conditions was consistent with this hypothesis. Initial rate kinetics and physical studies (using the techniques of gel chromatography, sedimentation velocity and sedimentation equilibrium) provided convincing evidence in support of the model. Although these studies could not eliminate the possibility of site-site interactions within the intact tetramer, the observed increase in specific activity upon dissociation of the enzyme to a dimer raises questions about the advantage nature would gain from these subunit interactions.

A comparison of the amino acid compositions of pig heart and *E. coli* succinyl-CoA synthetase revealed that the α subunits have very similar compositions except for an additional group of charged amino acid residues in the larger pig heart subunit. It was suggested that this difference may relate to the dissimilar oligomeric structures of the two enzymes. The physiological significance of the proposed dimer-tetramer equilibrium was discussed.

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ABBREVIATIONS AND SYMBOLS

ADP	adenosine-5'-diphosphate
ATP	adenosine-5'-triphosphate
CoA	Coenzyme A
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetate
GDP	guanosine-5'-diphosphate
GTP	guanosine-5'-triphosphate
HEPES	(N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid
MOPS	3-(N-morpholino)propane sulfonic acid
NADH	nicotinamide adenine dinucleotide
NBD-Cl	7-chloro-4-nitrobenzo-2-oxa-1,3-diazole
NEM	N-ethyl maleimide

Pc	partition coefficient
Pi	inorganic phosphate
SCS	Succinyl-CoA synthetase
Tris	tris-(hydroxymethyl)aminomethane

I. INTRODUCTION

Succinyl-CoA synthetase plays an important role in intermediary metabolism by participating in the citric acid cycle. The reaction that it catalyzes is reversible and relatively complex, with three substrates and three products:



In the reverse direction (as written in Eq. 1) the reaction represents the 'substrate-level' phosphorylation step of the tricarboxylic acid cycle. The forward reaction may be viewed as the nucleoside triphosphate dependent synthesis of succinyl-CoA and can be vital to microorganisms that require succinyl-CoA for biosynthetic purposes (1). Over the years, the two most popular sources of succinyl-CoA synthetase have been *Escherichia coli* and pig heart.

Interest in succinyl-CoA synthetase has been stimulated by an array of intriguing kinetic and structural features. The intricacies of its catalytic route are illustrated by the fact that a number of catalytic intermediates have been postulated, including an enzymic phosphohistidyl residue. More significantly for this dissertation, succinyl-CoA synthetase has been found to possess an interesting and relatively complex subunit structure. The enzyme from *E. coli* is believed to be a tetramer of the $\alpha_2\beta_2$ type while, in contrast, the enzyme from pig heart is considered an $\alpha\beta$ dimer. Attempts to rationalize these subunit structures form the basis of this thesis. Before further discussing the case

of succinyl-CoA synthetase, however, I wish to examine some ideas that have been proposed about the rationalization of the oligomeric structure of enzymes in general. (For a review on succinyl-CoA synthetase see Ref. 2).

A. *Why do enzymes have subunits?*

"Nothing in biology makes sense except in the light of evolution."

Although a large part of early work on proteins concerned monomeric enzymes, the existence of many oligomeric enzymes is now well documented (3). These enzymes may have either homologous subunit structures (i.e. the subunits are identical in primary sequence) or heterologous subunit structures (i.e. the component polypeptides are different). In view of the theory of evolution, these quarternary structures should have some general benefit in the design of proteins. Following is an examination of a number of different reasons that have been suggested for the existence of subunits.

One plausible rationale is that each subunit in an enzyme that contains different subunits may catalyse a portion of the overall reaction. For example, *E. coli* tryptophan synthase contains separate α and β_2 subunits that catalyze the cleavage of indole glycerol phosphate and the

¹Theodosius Dobzhansky, (1973) American Biology Teacher 35, p. 125.

condensation of indole with serine, respectively. It is not clear whether this enzyme can be regarded as having one active site at the subunit contact region (both catalytic activities being juxtaposed) or whether it has two separate active sites with the indole intermediate being channeled between the two regions (4). However, in the case of *Bacillus stearothermophilus* phosphofructokinase it appears that the active site is actually created through subunit interaction with the binding sites for substrates and allosteric effectors being located at the interface between the homologous subunits (5). Evidence will be reviewed later demonstrating that an isolated subunit of succinyl-CoA synthetase can catalyse a partial reaction and that the active site is at the subunit contact region.

It has also been proposed that the association of subunits in a multimeric enzyme may result in a more effectively folded conformation than that found in the corresponding monomer. In some cases this conformational change results in a more stable structure. For example, the monomeric form of aldolase (whether observed as an immobilized subunit on a matrix, as a transient subunit during refolding, or as a subunit stabilized through chemical modification) is more sensitive to inactivation by urea than the intact enzyme (6). The stabilized conformation resulting from subunit association may be reflected in an increased susceptibility of the isolated subunits to proteolysis. This was found to be the case with tryptophan synthase (7).

Another related phenomenon is demonstrated by this same enzyme. That is, a partial reaction catalyzed by one subunit may be stimulated in the presence of its partner (4). The formation of the complex thus appears to make the subunit a more efficient catalyst. It will be shown that succinyl-CoA synthetase also exhibits this property.

One of the more interesting aspects of the importance of subunits pertains to regulatory properties. The finding that there may be no steric resemblance between an inhibitor and the substrate of a regulatory enzyme led Monod *et al.* (8) to suggest that these enzymes must possess a site distinct from the active site. They labeled such sites 'allosteric' (allo=other). This concept leads to a crucial question: Does the subunit that binds the regulatory effector have to be distinct from the catalytic subunit? Aspartate transcarbamylase has been found to have two types of polypeptide chains, catalytic (c) and regulatory (r) (for review see (9)). In this case it appears that a separate subunit is needed to furnish the catalytic subunit with regulatory properties. Although historically this enzyme has served as a prototype for characterization of allosteric phenomena, because aspartate transcarbamylase is a heteromeric enzyme it differs from nearly all other regulatory enzymes. Glycogen phosphorylase (10) and phosphofructokinase (11) are just two examples of regulatory enzymes with homologous subunit structures. In these enzymes the allosteric effectors and the substrates bind to distal regions on the

same polypeptide chain. It would seem plausible that their activities could be modulated through interactions with the allosteric site in the monomer. The question may now be asked: Can strictly monomeric enzymes be allosteric? The idea that regulatory enzymes must possess more than one subunit has been firmly entrenched in enzymologists' minds. This dogma originated with the concerted transition theory put forward by Monod *et al.* (12). However, at least two allosteric enzymes have now been shown to be monomeric *in vitro*. These are the ribonucleoside triphosphate reductase of *Lactobacillus leichmannii* (13) and homoserine transacetylase from *Bacillus polymyxa* (14). It appears then that there is no compelling reason to believe that allosteric enzymes must always be polymeric.

The occurrence of a monomeric allosteric enzyme must however, be considered unusual, because such a protein lacks the physiological advantages of cooperativity. Homotropic cooperativity refers to the phenomenon in multisubunit proteins in which the binding affinity of a ligand changes as a function of ligand saturation. Because cooperativity has been found to arise from the interactions between subunits of oligomeric enzymes, a monomeric protein cannot exhibit cooperative effects (unless multiple binding sites are contained in a single polypeptide - e.g. the calcium binding sites in parvalbumin). The binding affinity may either increase (positive cooperativity) or decrease (negative cooperativity) as the binding sites are filled.

What advantage does cooperativity lend to multisubunit enzymes? It appears that cooperativity is a device for enhancing or dampening responses. Positive cooperativity provides increased sensitivity to the fluctuation of a ligand since the sigmoidal initial velocity plot rises more steeply than the non-cooperative hyperbolic curve. This would enable strategic control enzymes to respond to minor fluctuations in a highly controlled substrate, e.g., ATP. Negative cooperativity desensitizes an enzyme toward fluctuations in a ligand's concentration since the initial velocity plot is less steep than a Michaelis-Menten curve. Enzymes that should never be completely turned off could maintain a relatively constant activity in the midst of physiological changes. For regulatory proteins, allosteric effectors could in principle accelerate or decelerate an enzyme that exhibits no cooperativity. However, because of the subunit nature of the protein, cooperativity allows these same ligands to induce a more or a less pronounced effect than would be observed with independent sites. Therefore, this would appear to be the reason why virtually all regulatory proteins are found to have oligomeric structures. In the case of cooperative non-allosteric enzymes, their subunit structures would allow their activities to have increased or decreased sensitivity to environmental changes through homotropic site-site interactions alone. The difficulty that Monod *et al.* (8) had in justifying the high incidence of oligomeric structures in nonregulatory enzymes

may be explained by the advantages of cooperativity.

Recently it has become apparent that negative cooperativity can occur in an extreme form, i.e., half-site reactivity (15). It has been found that a large number of oligomeric enzymes react with a ligand or a covalent modifier at only half of their potential reactive sites. A few of the better documented examples of enzymes exhibiting this property are glyceraldehyde-3-phosphate dehydrogenase (16), alkaline phosphatase (17) and interestingly enough succinyl-CoA synthetase (18). Such a phenomenon poses the inevitable question: What advantage is gained from this extreme form of negative cooperativity? The net result of 'half-of-the-sites catalysis' is unimpressive from a kinetic point of view. When compared to the monomeric state, it amounts to improving the affinity for a substrate by a factor of two (because the dimer has two chances to combine with the substrate) while reducing the maximum velocity by the same factor (because half of the catalytic sites are shut down). A more subtle rationale for this extreme case of negative cooperativity has recently been developed. The general term that may be applied to the concept is 'catalytic cooperativity'. Similar, but less general theories, have been enunciated by Harada and Wolfe (19) as the 'reciprocating mechanism' and then later by Lazdunski (20) as the 'flip-flop mechanism'. The term 'alternating site cooperativity' has also been used (21). The essential feature of 'catalytic cooperativity' is that interaction of substrate at one site

in a multisubunit enzyme accelerates catalytic events at the other site. It need not be implied that the subunits alternate between the two functional states. However, use of the other terms suggests an additional feature whereby the two identical sites participate in catalysis in sequence. Be that as it may, if these interactions increase flux through rate-limiting steps then the catalytic efficiency of the subunit will increase. An increase in catalytic efficiency of several fold would more than compensate for the fact that half of the protein sites may be unavailable for direct catalysis. Therefore, one of the most important roles of negative cooperativity could be the improvement of the catalytic efficiency of one subunit through conformational changes promoted by the ligand-binding energy of the other.

Convincing evidence for catalytic cooperativity has been difficult to obtain since, in the normal substrate concentration range, Michaelis-Menten kinetics prevail. However, since binding of substrate to one subunit modulates intermediate reaction steps on an alternate subunit, certain intermediate reactions should be dependent on substrate concentration. This effect offers a potential way of detecting catalytic cooperativity. Recently a number of studies have revealed substrate modulations of oxygen exchange to occur in ATP synthesis by mitochondria and chloroplasts (22,23,24), with membrane-bound ATPase (25), with soluble mitochondrial ATPase (26,27), with glutamine synthetase (28) and finally, with *E. coli* succinyl-CoA

synthetase (29). In each of these studies it has been proposed that the most probable explanation of the results is catalytic cooperativity. That is, binding of the substrate to a catalytic site of one subunit affects the intermediate steps that result in oxygen exchange on the other subunit. A major part of this thesis is involved with a further investigation into the mechanism of catalytic cooperativity in succinyl CoA synthetase.

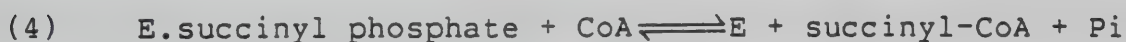
It must be borne in mind that another school of thought maintains that there is no evidence that the phenomenon of half-site reactivity is of general importance in the normal catalytic turnover (30). In this vein, recent studies on *E. coli* alkaline phosphatase using alternative substrates as a probe for catalytic cooperativity indicate that this mechanism, despite its attractiveness, does not seem to operate in that enzyme system (31).

The preceding discussion has focused on situations where the structure or function of the enzyme molecule itself has benefited from its oligomeric structure. For example, it has become more stable, more sensitive to regulation, or more catalytically efficient. Possibly one should think in broader terms when attempting to justify subunit structures to determine if there are any rationales in which the cell or organism as a whole appears to gain the advantage by having its enzymes largely in oligomeric form. A number of different reasons of this nature have been suggested including reduction of osmotic pressure and

facilitation of compartmentation. These rationales are less obvious than the previously discussed ones, and are much more difficult to assess experimentally. Perhaps no general answer to the question of the significance of subunit structures exists. It may be that each oligomeric structure is '...merely a reflection of the haphazard way in which that great opportunist, Natural Selection, arrives at an effective solution...' (32) to the problems of catalysis and regulation.

B. Succinyl-CoA synthetase background

It is presently thought that the overall reaction catalyzed by succinyl-CoA synthetase occurs in three steps:



The first partial reaction involves the formation of the phosphoenzyme intermediate (E-P) in which a histidine residue is phosphorylated. The suggestion of a phosphorylated derivative of succinyl-CoA synthetase was first made by Kaufman (33) who detected an isotope exchange reaction between ADP and ATP in the presence of spinach succinyl-CoA synthetase and Mg^{2+} . A similar suggestion was made for the *E. coli* enzyme when it was observed to covalently incorporate label from $[\gamma\text{-}^{32}P]ATP$ but not from $[8\text{-}^{14}C]ATP$ (34). This led to the identification of phosphohistidine in

alkaline digests of *E. coli* succinyl-CoA synthetase (35).

The mere existence of a phosphorylated derivative of an enzyme does not prove that it is an obligatory intermediate in catalysis - for example, the phosphorylation could be structural or serve a regulatory role. However, experiments using rapid mixing and quenching techniques showed that the presteady state kinetics of the phosphorylated form of succinyl-CoA synthetase satisfied the criteria expected of an obligatory intermediate; i.e., the steady state level of E-P is reached before the overall reaction achieves steady state and the initial rate of E-P formation is faster than the steady state rate of the overall reaction (36).

The first suggestion that succinyl phosphate might participate as the second catalytic intermediate was based on the observation that ^{18}O is transferred from succinate to phosphate during catalysis by either the bacterial enzyme (37) or the mammalian enzyme (38). This is one of the most important facts concerning the mechanism of succinyl-CoA synthetase, especially with regard to the subject of this thesis, since it is this oxygen exchange reaction that is exploited as a probe for catalytic cooperativity in succinyl-CoA synthetase. The most convincing evidence for the existence of the succinyl phosphate intermediate was reported by Nishimura and Meister (39) who showed that:

1. [^{32}P] succinyl phosphate could be separated from reaction mixtures containing succinate, [γ - ^{32}P] ATP, and high concentrations of the bacterial enzyme.

2. Succinyl phosphate reacted with ADP to produce ATP and with CoA to produce succinyl-CoA in the presence of the enzyme.

3. [^{32}P] succinyl phosphate reacted with the enzyme to form [^{32}P] phosphoenzyme and in the reverse direction phosphoenzyme and high concentrations of succinate reacted to form succinyl phosphate.

The observation that the rate of dephosphorylation of the enzyme by succinate is much slower than the overall reaction led to the suggestion that succinyl phosphate may not be a discrete intermediate but that, alternatively, reaction (3) and (4) may occur in a concerted fashion (40). This mechanism would still account for transfer of ^{18}O between succinate and phosphate. These doubts about the succinyl phosphate intermediate were largely dispelled by the findings that desulpho-CoA stimulates the production (41) and utilization (42) of succinyl phosphate.

These stimulatory effects of desulfo-CoA are related to an intriguing feature of succinyl-CoA synthetase catalysis termed 'substrate synergism'. The finding that the ADP - ATP exchange reaction catalyzed by the bacterial enzyme is enhanced in the presence of other substrates led to the concept of substrate synergism - that is, the active site is fully active only when all substrate binding sites are filled (36). These effects could be the result of conformational changes in the locality of the active site. Other manifestations of substrate synergism are ATP stimulation of

the succinate - succinyl-CoA exchange and CoA stimulation of the ATP - E-P exchange (36). Substrate synergism may be a general property of the enzyme from a variety of sources, since CoA and succinate stimulate the GDP - GTP reaction catalyzed by the heart enzyme (43).

The subunit structure of succinyl-CoA synthetase was elucidated in the 1970's and is of primary significance to this thesis. Although it had been previously noted that the enzyme seemed to dissociate in the presence of either mercurials (44,45) or denaturants (46), less equivocal evidence for the presence of subunits was provided by Bridger (47) using the technique of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. Electrophoresis of the bacterial enzyme in the presence of detergent gives rise to two bands corresponding to two subunits of differing size. Their molecular weights were estimated to be approximately 30,000 and 39,000 for the smaller (α) and larger (β) subunits, respectively. It was suggested that the *E. coli* enzyme is a tetramer of the $\alpha_2\beta_2$ type. The predicted molecular weight of this structure would be near 140,000, which agrees nicely with that determined for the native enzyme by sedimentation equilibrium techniques (44,48).

As was the case for the bacterial enzyme, a preparation of pig heart succinyl-CoA synthetase gave rise to two discrete bands when subjected to electrophoresis in the presence of detergent (49). Their respective molecular weights were estimated to be 42,500 (β) and 34,500 (α). The

molecular weight of the native heart enzyme has been estimated to date only by gel filtration on calibrated columns, but all determinations agree fairly well with a value of 75,000 (49,50,51). These results strongly suggest that the pig heart enzyme is a dimer of the $\alpha\beta$ type.

It emerges that one striking difference between *E. coli* and pig heart succinyl-CoA synthetase is in their molecular weights; the pig enzyme is approximately half the size of the *E. coli* enzyme. The molecular weights of three other bacterial succinyl-CoA synthetases have been reported to be very similar to that of the *E. coli* enzyme (52). A recent survey based on gel filtration behaviour indicates that succinyl-CoA synthetases from a variety of sources may be divided into 'large' (corresponding to molecular weights of 140,000) and 'small' (molecular weights of 70,000) types (53). Only Gram-negative bacteria produce the 'large' enzyme while Gram-positive bacteria and diverse eukaryotic organisms produce the 'small' type. Two questions can be formulated when contemplating these intriguing quarternary structures of succinyl-CoA synthetase and they are discussed below.

C. Why two kinds of subunits?

Studies done on other heteromeric enzymes (some examples are described in the first part of this chapter) have indicated that each subunit type may have a different

function. To assign roles to the α and β subunits of succinyl-CoA synthetase methods were developed for their separation so that each could be studied in isolation. Pearson (54) achieved this using gel filtration in the presence of 6 M urea and 5% acetic acid. They also discovered that, in the presence of ATP, the isolated subunits may be reassembled to form active enzyme. These techniques of subunit isolation and recombination have been exploited in some of the studies presented herein. However, detailed characterization of the isolated subunits was hampered by the finding that they are only slightly soluble in benign buffers.

Previous to the isolation of the subunits it was determined, using gel electrophoresis in the presence of detergent, that the smaller α subunit is the site of phosphorylation after incubating the enzyme with [32 P] ATP (47). By testing the purified subunit, it was discovered that α is capable of catalyzing its own phosphorylation by ATP (55). This result establishes that, not only is the phosphohistidine residue in the α subunit, but also the ADP and ATP binding sites together with the equipment for phosphoryl transfer. In this regard succinyl-CoA synthetase is much like the previously discussed example of tryptophan synthase - that is, an isolated subunit of each enzyme catalyzes a partial step in its overall reaction. Also resembling the case of tryptophan synthase is the observation that the presence of β speeds up the rate at which α phosphorylates

itself (55). It thus appears that β can promote a conformational change in α which makes the latter a more efficient catalyst.

This is not the only role which has been suggested for the β subunit. Unlike the holoenzyme, the α subunit does not undergo phosphorylation in the presence of succinyl-CoA and Pi (the reverse of reactions 3 and 4). This was the first indication that the binding sites for succinate and CoA might be located on the β subunit. An observation that lends support to this concept is the syncatalytic succinylation of a lysine residue in β (55). During the course of my studies, more compelling evidence for the CoA site being located on the β subunit was reported by Nishimura and Collier (56), who demonstrated affinity labeling of the β subunit by oxidized CoA-disulfide. Using the approach of affinity chromatography, I have gained further evidence that CoA binds to the β subunit. From all of this, the active site may be visualized at the contact point between the two subunit types. Thus, the rationale for the presence of two types of subunits in succinyl-CoA synthetase seems to be that both are required for assembly of the active site, with ATP and ADP binding to α and succinate and CoA interacting with β .

D. Why two copies of each subunit per molecule?

Evidence has been presented suggesting that the quaternary structures of succinyl-CoA synthetase molecules from a variety of sources may be divided into two categories. The Gram-negative bacteria (*E. coli* included) produce the 'large' enzyme ($\alpha_2\beta_2$ tetramers) while Gram-positive bacteria and eukaryotes produce the 'small' type ($\alpha\beta$ dimers). The following discussion concerns possible rationales for the presence of two copies of both α and β in the *E. coli* enzyme molecule.

There are several indications that the two halves of the *E. coli* succinyl-CoA synthetase molecule are capable of communicating with each other. It has been observed that the enzyme shows half-of-the-sites reactivity with respect to phosphorylation - that is, only one phosphoryl group is incorporated per $\alpha_2\beta_2$ tetramer (44,57). In contrast, a stoichiometry of phosphorylation approaching two has also been reported (58). More recently, the issue has been reinvestigated by Bowman and Nishimura (59). They used a chromatographic technique to show that *E. coli* enzyme of low specific activity incorporated approximately 1 mole of phosphate per tetramer while enzyme of high specific activity incorporated about 1.7 moles of phosphate. An NMR relaxation time study on the Mn(II) ion complex of the *E. coli* succinyl-CoA synthetase molecule has lent support to this concept by demonstrating that the enzyme of high specific activity has four metal ion binding sites with

indistinguishable dissociation constants while the enzyme of lower specific activity has two strong metal binding sites and two weak metal binding sites (60). However, a very recent study carried out in our laboratory, making use of the techniques of subunit isolation and recombination, indicates that a hybrid enzyme species containing only one phosphorylated α subunit can be prepared (61). As discussed in Levitzki and Koshland's review on negative cooperativity and half-site-reactivity this type of inconsistent behaviour '...may well be a pattern for half-of-the-sites reactivity in many proteins when they are investigated in sufficient detail' (15). That is, the conformational changes induced by the interaction of the first ligand may cause the remaining half of the molecule to be unreactive but not inert. Therefore, depending on the experimental conditions, essentially stoichiometric amounts of monosubstituted enzyme may be formed before significant amounts of disubstituted enzyme are obtained. Whether *E. coli* succinyl-CoA synthetase exhibits complete half-of-the-sites reactivity or merely strong negative cooperativity is immaterial to the following proposal. The important idea is the implied communication between the two active sites.

This sort of communication indicates that prominent interactions between subunits must occur. Boyer and his co-workers have proposed that these interactions raise the possibility of the operation of catalytic cooperativity in this system. They have interpreted their data on the

modulation of oxygen exchanges catalyzed by succinyl-CoA synthetase to be supportive of this concept. Further, they suggest that the *E. coli* enzyme operates with its two identical active sites participating in sequence such that acceleration of catalytic events at one site results from interaction of a substrate at the other site (29). A major part of this thesis involves a detailed investigation into the validity and possible mechanism of catalytic cooperativity in *E. coli* succinyl-CoA synthetase using in part the elegant method of analysis developed by Bild, Janson and Boyer. Many of the results were unexpected but are indicative of the complexity and capricious nature of this enzyme system.

II. STUDIES ON THE ACTIVE SITE OF E. COLI SUCCINYL-CoA SYNTHETASE

A. Introduction

Studies done on oligomeric enzymes containing more than one kind of subunit have revealed that each subunit type may have a different function and, in some cases, that the active site may be assembled at the subunit interface. Evidence was reviewed in the Introduction indicating that this may be the case for succinyl-CoA synthetase, i.e. since the α -subunit is capable of catalyzing its own phosphorylation from ATP but not from succinyl-CoA and phosphate it was proposed that ATP binds to the α subunit while the CoA and succinate binding sites are located on the β subunit, or comprise part of both subunit types. I have attempted to establish the location of the CoA site more firmly by studying the binding of the isolated subunits of *E. coli* succinyl-CoA synthetase to an agarose-hexane-coenzyme A affinity resin. Although the results of this study were complicated by the insolubility of the isolated subunits, they support the concept that the CoA binding site may be on the β subunit.

Another approach involved the modification of intact *E. coli* succinyl-CoA synthetase. Chemical modification techniques have shown that arginine residues, largely protonated at physiological pH, serve as recognition sites for anionic ligands in many proteins (62). It seemed reasonable to

expect that arginine residues might be involved in succinate, CoA and/or ATP binding in succinyl-CoA synthetase. The following experimental design was developed: if specific arginine residues located in a binding site could be protected from modification by substrate, then that protected group could be reacted with [14 C] phenylglyoxal (an arginine-selective α dicarbonyl (63)) after substrate removal and isolation of the modified subunits would reveal the location of the binding site. It will be shown that phenylglyoxal does inactivate *E. coli* succinyl-CoA synthetase. However, protection by substrates was not considered sufficiently complete to warrant further studies involving the isolation of the modified subunits.

A second modification study was done on *E. coli* succinyl-CoA synthetase. This involved the reaction of sulfhydryl groups with NBD-Cl (7-chloro-4-nitrobenzeno-2-oxa-1,3-diazole), a fluorogenic probe first reported by Birkett *et al.* (64). Hybrid enzymes were created by reconstituting normal and modified subunits. The properties of these molecules revealed that sulfhydryl groups in both the α and β subunits are important for activity.

B. Materials and Methods

1. Purification and Storage of succinyl-CoA synthetase

The general methods described here were used in the preparation and handling of succinyl-CoA synthetase throughout the study reported in this dissertation. The *E.*

coli enzyme was purified from Crooke's strain of *E. coli* grown on succinate-based medium by methods described earlier (46,65). The pig heart enzyme (which is studied in some of the latter chapters) was purified from fresh pig hearts essentially by the procedure of Cha *et al.* (66), with addition of a final purification step involving affinity chromatography on Blue Sepharose CL-6B. Preparations were judged to be pure by homogeneity upon gel electrophoresis. The concentrations of both enzymes were routinely determined from their absorbance at 280 nm (48,67). Various preparations of the purified *E. coli* enzyme had specific activities ranging from 35 to 45 units/mg when assayed by the direct spectrophotometric method (68). Using a similar assay medium (66), the pig heart enzyme exhibited a specific activity of approximately 20 units/mg. (Other assay systems were employed in this thesis and they are described in the pertinent sections). Both the *E. coli* and pig heart enzyme were stored at 4°C as precipitates in 70% saturated ammonium sulfate. Small amounts of the enzyme were centrifuged, dialyzed extensively against the appropriate buffer, and then run through a small gel filtration column to remove any remaining salt prior to each experiment.

2. Affinity Chromatography

The agarose-hexane-coenzyme A resin (AgCoA Type 1 from P.L. Biochemicals, Inc.) contained 0.5 μ moles of CoA per ml of packed wet gel. It was prepared by reacting CoA with an agarose-hexanoic acid resin using dicyclohexylcarbodiimide

so that the final product contained the free sulfhydryl group on the CoA moiety. The agarose hexanoic acid resin (Aghexanoic from P.L. Biochemicals, Inc.) was used as the control matrix. Small columns (prepared in pasteur pipettes) containing 1 ml of the CoA or control resin were equilibrated with a 20 mM Tris-HCl buffer, pH 7.0 containing 0.75 M urea. The urea was included in the elution buffer in order to keep the isolated subunits from precipitating out of solution. Even in the presence of the urea the solubility of the subunits was limited and it was necessary to work with very dilute solutions. Because of the low protein concentrations involved, the subunits were detected as they came off the columns by the sensitive protein-dye binding method of Bradford (69), whereby the binding of Coomassie Brilliant Blue G-250 to protein causes a shift in the absorption maximum of the dye from 465 to 595 nm, and the increase in absorption at 595 nm is monitored. The original concentration of the α and β preparations was also determined using this technique.

The isolated α and β subunits were prepared from *E. coli* succinyl-CoA synthetase by gel filtration according to the methods developed by Pearson and Bridger (54). Samples (300 μ l containing approximately 200 μ g of protein) of either the α or β subunits were applied to the CoA and control columns and 1 ml fractions were collected at a flow rate of 0.2 ml/min. The columns were first eluted with 10 ml of the Tris-urea buffer, and then with another 10 ml of the

same buffer containing 0.6 M KCl.

Tris (ultra pure) and urea were obtained from Schwarzmann. The Coomassie Brilliant Blue G-250 was purchased from Serva.

3. Arginine Modifications

E. coli succinyl-CoA synthetase was modified by incubating 75 to 200 μ g of enzyme with 4.0 μ mole of phenylglyoxal in 250 μ l of 50 mM MOPS buffer, pH 8.2 at 25°C. The kinetics of the inactivation were followed by taking aliquots of the incubation mixture at appropriate time intervals and assaying for activity by dilution into a modified direct assay solution which contained MOPS instead of the standard Tris buffer (68). The enzyme was phosphorylated prior to inactivation by incubating it for 15 minutes at 25°C in a solution containing 50 mM Tris-HCl pH 7.2, 50 mM KCl, 5 mM MgCl₂, and 100 μ M ATP and then dialyzing it exhaustively against the MOPS buffer. In the protection experiments an excess of substrate was included in the incubation mixture together with the enzyme, phenylglyoxal and 2 mM MgCl₂. MOPS and phenylglyoxal were obtained from Sigma. ATP was purchased from Terochem (Edmonton, Canada) and CoA from P.L. Biochemicals.

4. NBD-Cl modifications

The *E. coli* enzyme was modified by incubating it, at a concentration of approximately 0.7 mg/ml, with NBD-Cl in 50 mM MOPS buffer, pH 7.2 at 25°C. In the kinetic experiments, a NBD-Cl concentration of 0.1 mM was used and

the rate of inactivation was monitored by assaying aliquots of the incubation mixture in the modified direct assay (68) containing MOPS buffer; the absorbance change was followed by incubating the mixture in a total volume of 1 ml in a cuvette in the spectrophotometer. In the experiments where the reaction was taken to completion an NBD-Cl concentration of 1.0 mM was used and all other conditions remained the same.

5. Preparation of NBD-Cl modified subunits

The enzyme (approximately 14 mg) in a total volume of 20 ml was modified with NBD-Cl (1.0 mM) until the reaction was complete, i.e. 16 sulfhydryl groups were modified in each enzyme molecule. Ammonium sulfate (10 grams) was added and the precipitated enzyme was spun down and resuspended in 2 ml of 50 mM MOPS, pH 7.2. Any remaining salt and unreacted NBD-Cl were removed by running 20 ml of the MOPS buffer through the sample in a small Amicon ultrafiltration apparatus (a YM-10 membrane was used). The protein was then transferred into the urea-acetic acid separating buffer by running 14 ml of this buffer through the ultrafiltration cell. The remaining 2 ml of modified enzyme were loaded onto a gel filtration column and the subunits were separated according to the methods previously described (54). NBD-Cl was purchased from Pierce Chemical Company.

C. Results

1. Affinity Chromatography

Preliminary experiments were done which showed that native *E. coli* succinyl-CoA synthetase would bind to a CoA-hexane-agarose resin and that it could be eluted with the addition of 0.6 M KCl to the buffer. It was also found that the native enzyme did not bind to the control hexane-agarose resin. Having done these background experiments, the binding of the isolated α and β subunits to the two columns was examined. It can be seen in Figure 1 that the majority of the β subunit binds to the CoA resin and is eluted when 0.6 M KCl is added to the buffer. It did not bind to the control column. Therefore, the β subunit mimics the behaviour of the intact enzyme. Unfortunately, the α subunit bound to both resins and could be eluted only with the addition of 0.6 M KCl.

2. Arginine Modifications

When *E. coli* succinyl-CoA synthetase was incubated with phenylglyoxal, the enzyme was progressively inactivated. The kinetics of inactivation was biphasic (Figure 2) with the rapid reaction occurring immediately upon exposure of the enzyme to phenylglyoxal, and the slow inactivation continuing until the enzyme was almost completely inactive at 50 minutes. Apparent rate constants were calculated for these two reactions using the method of Ray and Koshland (70), i.e. the final slope gives the rate constant for the slower reaction ($k=0.03 \text{ min}^{-1}$ and subtraction of the values

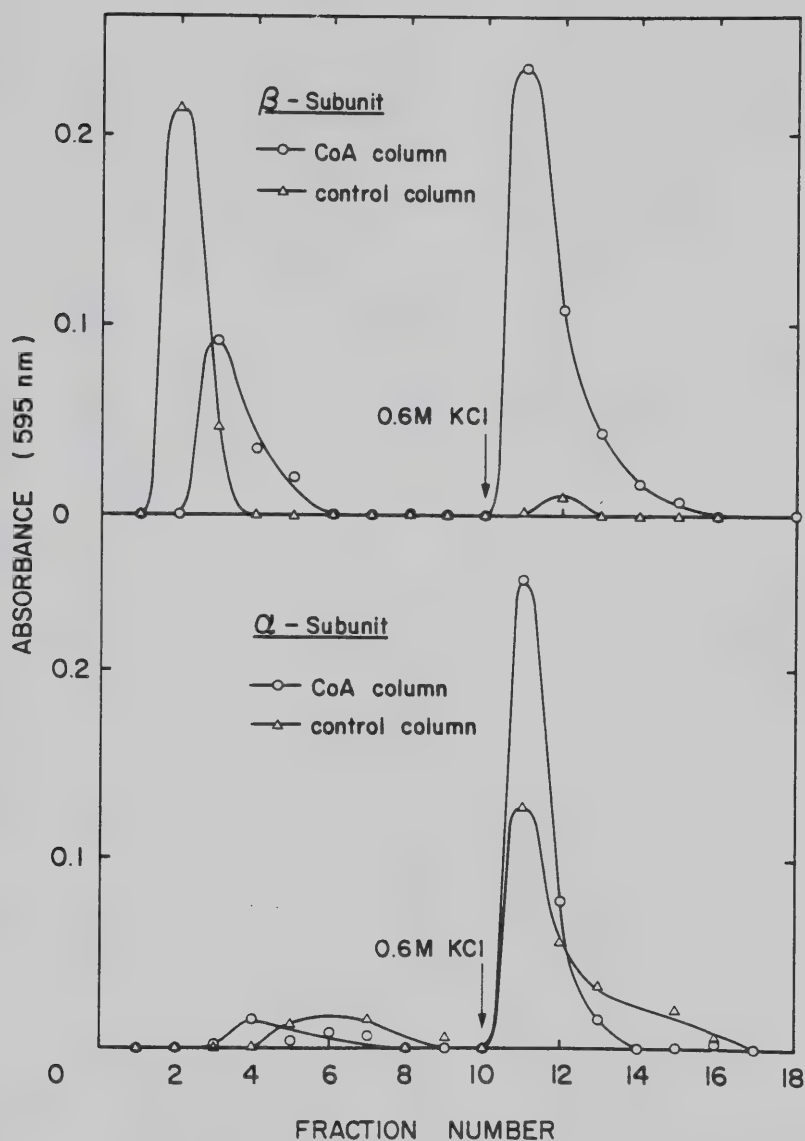


Fig. 1 - Affinity chromatography of the α and β subunits of *E. coli* succinyl-CoA synthetase. 200 μ g of the α or β subunit were loaded onto the CoA and control columns. The columns were first eluted with 10 ml of 20 mM Tris-HCl, pH 7.0 containing 0.75 M urea and then with 10 ml of the same buffer also containing 0.6 M KCl. Other experimental details are included in the text.

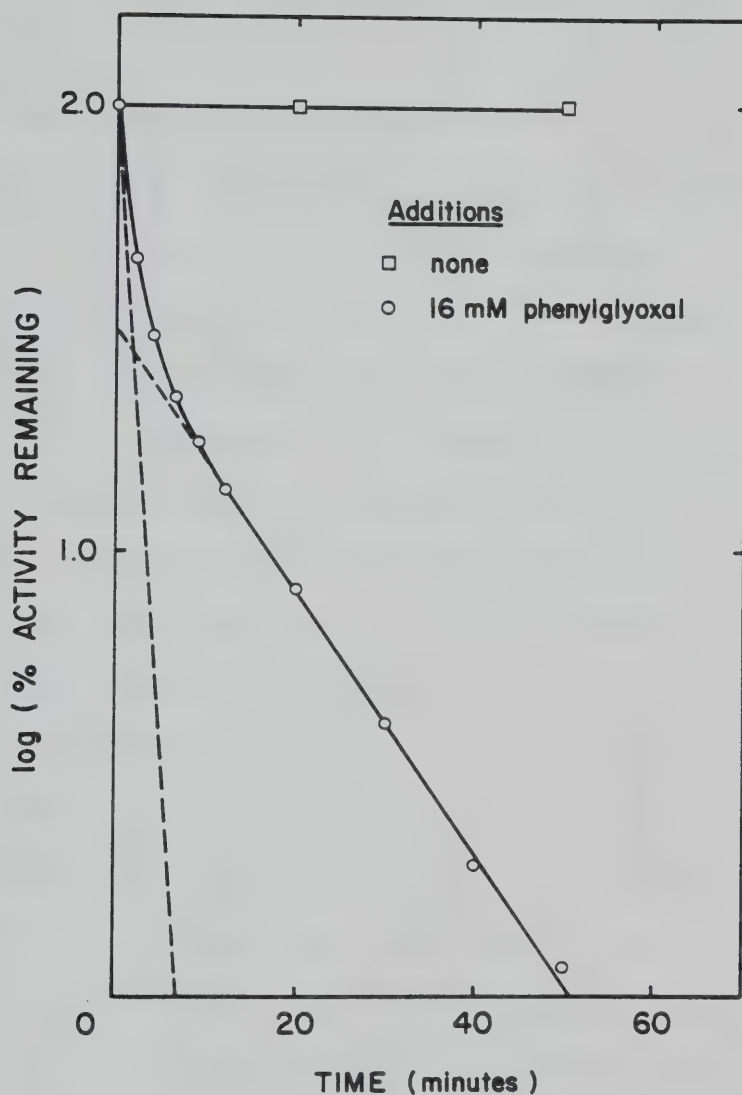


Fig. 2 - Inactivation of *E. coli* succinyl-CoA synthetase by phenylglyoxal. 95 μ g of *E. coli* enzyme, in 50 mM MOPS, pH 8.2 were incubated in a final volume of 250 μ l at 25°C with the additions shown. Aliquots were assayed for activity after the indicated time intervals. The values shown are the average of three determinations.

along the extrapolated slope from the observed values gives the rate constant for the rapid reaction ($k=0.27 \text{ min}^{-1}$). Thus, the rapid inactivation was 9-fold faster than the slow inactivation at this concentration of phenylglyoxal.

If this inactivation is attributable to phenylglyoxal modifying arginine residues that are involved in the binding of anionic substrates, then the presence of these substrates might be expected to protect the site from attack. Experiments testing this possibility are described in Figure 3. The enzyme was protected to some extent from both the rapid and slow inactivation by the presence of 5 mM ATP or 5 mM CoA. These two substrates together provided a slightly higher level of protection. It can be seen that succinate not only does not provide any protection, but enhances the susceptibility of the enzyme to inactivation. Since succinate promotes the dephosphorylation of the enzyme in the presence of Mg^{2+} (40), and thereby promotes formation of a "looser" conformation for the enzyme (18), it was reasoned that the increased susceptibility to phenylglyoxal brought about by succinate could be a reflection of the state of phosphorylation of the enzyme. This was confirmed by experiments in which the samples were incubated for increasing times with succinate prior to inactivation by phenylglyoxal for a set length of time. The rate of increase in susceptibility corresponded well with the rate reported for the succinate-dependent dephosphorylation reaction (18).

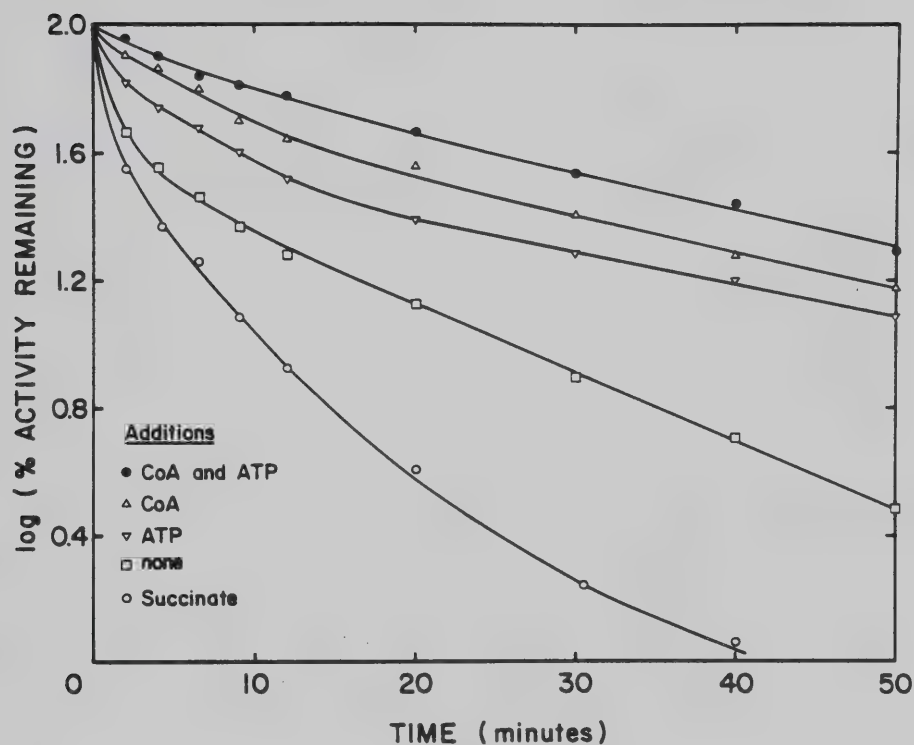


Fig. 3 - Protection by substrates against inactivation by phenylglyoxal. Approximately 200 μg of *E. coli* enzyme were incubated in a final volume of 250 μl of 50 mM MOPS, pH 8.2 which contained 5 mM Mg^{2+} plus the substrate additions shown (all substrates were at 5 mM). The results are the average of three determinations.

3. NBD-Cl modifications

The essential features of inactivation experiments using NBD-Cl are shown in Figure 4. In the absence of substrates there was a fairly rapid, pseudo-first-order inactivation of *E. coli* succinyl-CoA synthetase. Mg^{2+} , alone or together with ATP, retarded the rate of inactivation slightly with ATP adding no additional protection above Mg^{2+} alone. Addition of CoA and Mg^{2+} provided complete protection from inactivation but this is most likely attributable to reaction of NBD-Cl with the free sulfhydryl group on CoA causing the loss of its inhibitory properties.

This time-dependent inactivation was paralleled by an increase in absorbance at 420 nm. NBD-Cl is known to react specifically with free sulfhydryl groups in proteins at pH 7.0-8.0 (71,72) giving rise to a fluorescent derivative (S-NBD). The reaction product with -SH is identified by an absorbance maximum at 420 nm and an emission maximum at 520 nm. The excitation and emission spectra of the derivatized *E. coli* enzyme were examined and peaks at 420 nm and 520 nm were found. There was no evidence of an absorbance maximum at 475 nm which is characteristic of the reaction product with amino groups.

The relationship between the loss of activity and the number of sulfhydryl groups modified is shown in Figure 5. The stoichiometry of modification was calculated using an extinction coefficient of $13.0 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at 420 nm (72) for the S-NBD derivative. The data indicate that

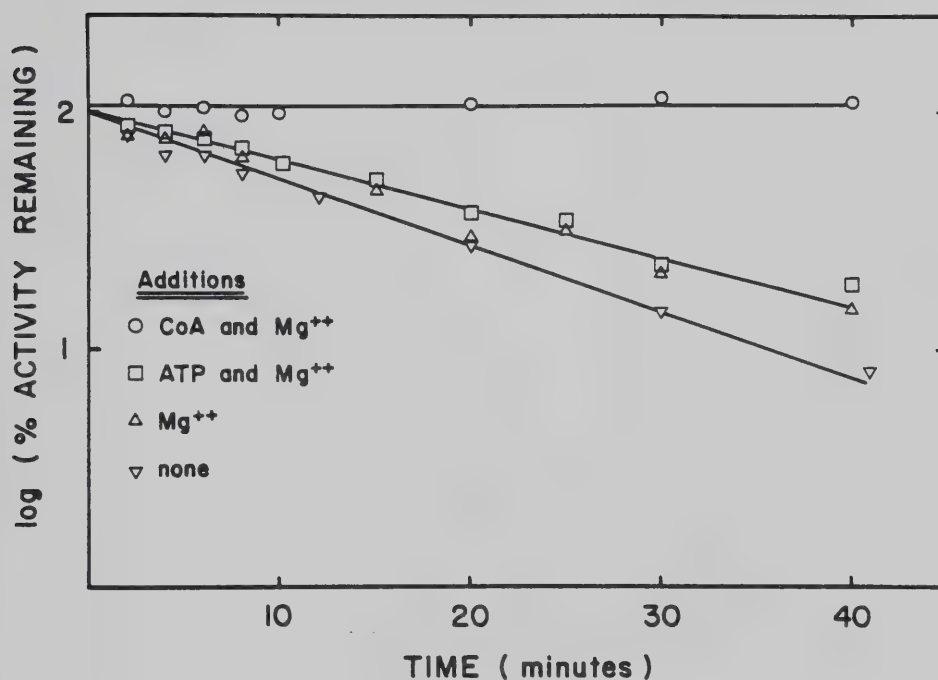


Fig. 4 - Protection by substrates against inactivation by NBD-Cl. The reaction mixtures contained 50 mM MOPS buffer, pH 7.2, 120 μ g enzyme, 0.4% ethanol, 20 nmole NBD-Cl in a total volume of 256 μ l. CoA, ATP and/or Mg²⁺ were added to a final concentration of 5 mM where indicated. The mixture was incubated at 25°C and samples were removed at the indicated times and assayed. Ethanol was used as a solvent for NBD-Cl.

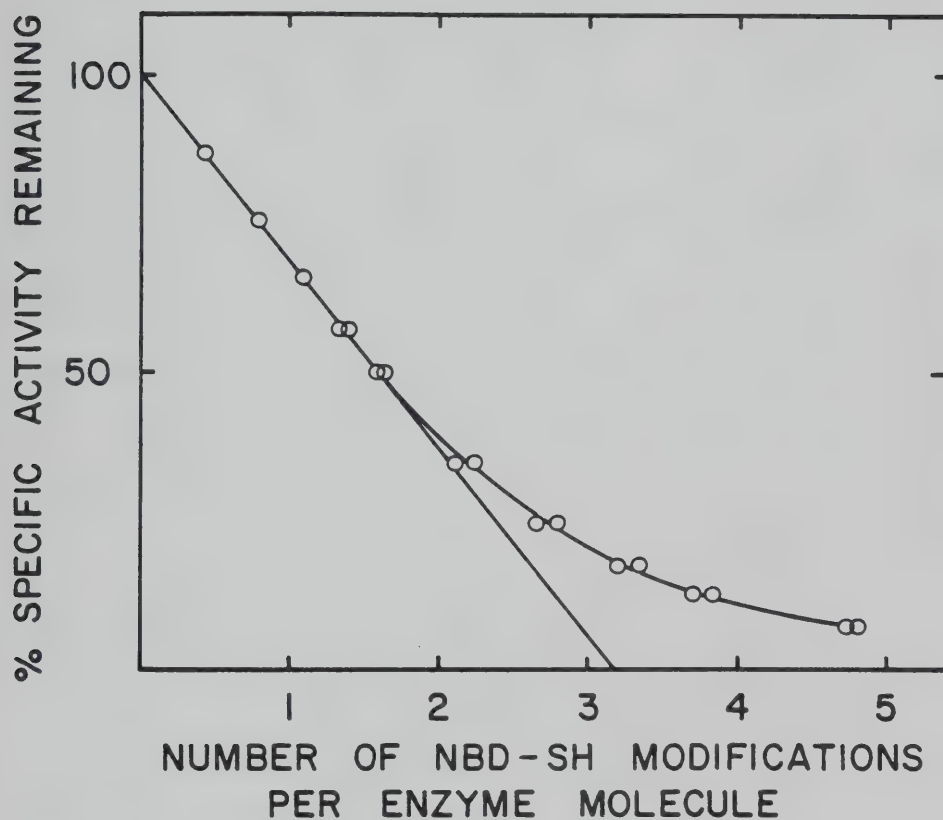


Fig. 5 - Titration of *E. coli* succinyl-CoA synthetase with NBD-Cl. The reaction mixture contained 50 mM MOPS buffer, pH 7.2, 740 μ g enzyme, 0.5% ethanol, 100 nmole NBD-Cl in a total volume of 1.0 ml. When following the absorbance change the mixture was incubated at 25°C in the spectrophotometer. To measure the inactivation, aliquots from a duplicate mixture were assayed.

modification of approximately 3 important sulfhydryl groups is involved in the total inactivation of *E. coli* succinyl-CoA synthetase. Even after the enzyme is almost completely inactivated the number of sulfhydryl groups being modified continues to increase. Experiments were done to determine the total number of sulfhydryl groups available for modification (the concentration of NBD-Cl was increased 10-fold) and the results shown in Figure 6 indicate that of the total of 24 sulfhydryl groups on the enzyme (54), a maximum of 16 sulfhydryl groups could be modified.

When the α and β subunits of the fully modified enzyme were separated and the number of modified sulfhydryl groups per subunit was determined, again using the known extinction coefficient for the derivative, it was found that each subunit retained approximately 4 modified groups. This adds up nicely to the original 16 modifications in the intact $\alpha_2\beta_2$ tetramer. Experiments were performed employing the techniques previously developed by Pearson (54) for recombination of normal α and β subunits into active enzyme. It was found that no detectable enzyme activity was regenerated when the NBD-Cl-modified α and normal β or modified β and normal α were recombined together. It was anticipated that the derivatized subunits were actually refolding with the unmodified subunits in these experiments, since the sulfhydryl groups involved in subunit contacts should have been protected during the modification of the intact enzyme. This was corroborated by the finding that either modified subunit

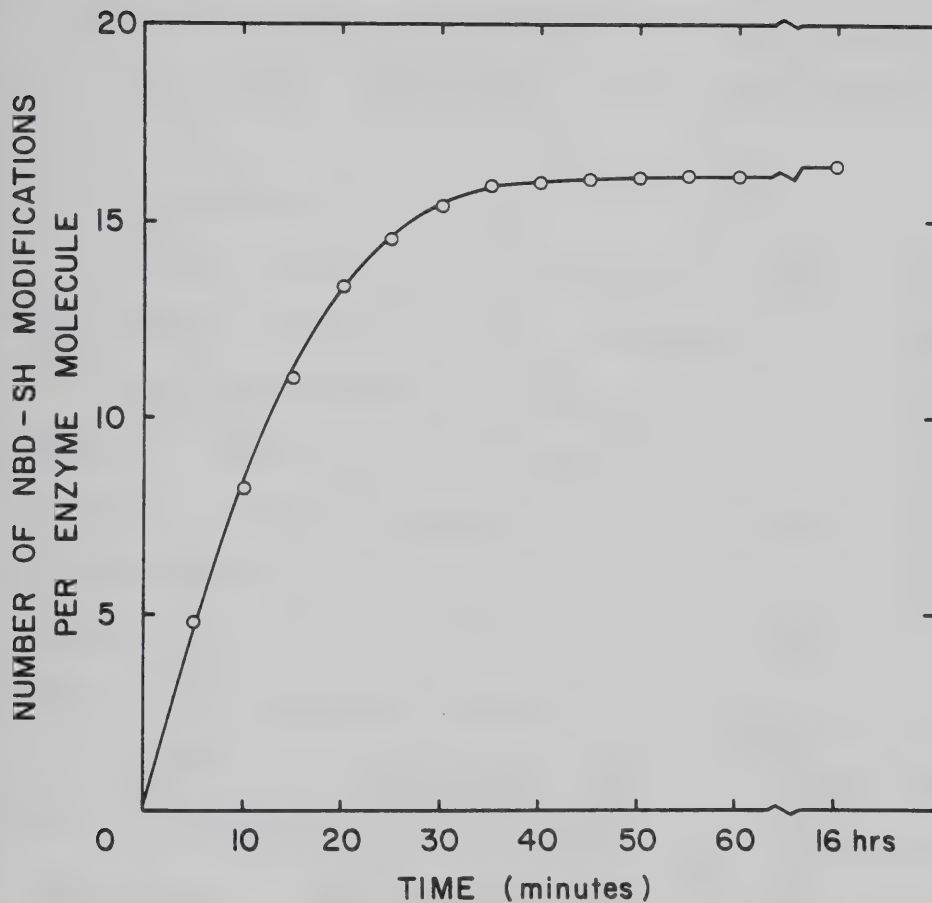


Fig. 6 - Maximum modification of *E. coli* succinyl-CoA synthetase with NBD-Cl. The reaction mixture contained 50 mM OPS, pH 7.2, 735 μ g enzyme, 4.8% ethanol, 1.0 μ mole NBD-Cl in a total volume of 1.05 ml. The increase in absorbance was monitored by incubating the mixture at 25°C in the spectrophotometer.

is able to reduce the specific activity of the refolded enzyme, even when stoichiometric amounts of normal α and β are present, i.e. the modified subunit appears to compete with the normal subunit for its partner during refolding.

D. Discussion

The affinity chromatography studies using the CoA-hexane-agarose resin indicate that the isolated β subunit may contain an intact CoA binding site since the subunit binds to the CoA resin but not to the control column (Figure 1). Unfortunately, it is difficult to make firm conclusions about the CoA binding ability of the α subunit from these experiments since it is bound to both the CoA- and control columns (Figure 1). This binding is probably due to non-specific hydrophobic and ionic forces contributed by the spacer-arm matrix assemblies. Subsequent to these experiments, affinity labelling studies in Nishimura's laboratory have lent support to the concept of the CoA site being on the β subunit. Incubation of oxidized CoA disulfide (73) or S-(4-bromo-2,3-dioxobutyl) CoA (74) with *E. coli* succinyl-CoA synthetase led to the formation of inactive enzyme and sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that the site of labelling was on the β subunit. Previous to these affinity chromatography and labeling experiments it had been speculated that the binding site for CoA might lie on the β subunit since its presence was required to accomplish phosphorylation of the α subunit

from succinyl-CoA and Pi (55). The affinity chromatography results show that a functional CoA binding site resides on the β subunit. Thus, the β subunit appears to have a catalytic role and the complete active site must be assembled at the $\alpha\beta$ interface.

The inactivation of *E. coli* succinyl-CoA synthetase by phenylglyoxal showed biphasic kinetics (Figure 2). As discussed by Ray and Koshland (70), this may be a reflection of several residues being modified with different rates, enzyme heterogeneity or progressive denaturation. It is unlikely that the biphasic inactivation is related to the presence of phospho- and dephospho- forms of the enzyme (as was the case when the rat liver enzyme was inactivated with dial-GDP (75)) since the enzyme was purposely phosphorylated prior to inactivation and, secondly, when ATP-Mg²⁺ was included during the incubation it did not selectively protect against either phase of the inactivation. The fast and slow inactivation may be due to the presence of dimer and tetramer in solution (evidence will be given for this equilibrium in later chapters), but with the use of [¹⁴C]-phenylglyoxal, Nishimura has shown (personal communication) that 3 arginine groups per $\alpha\beta$ dimer (6 per tetramer) are important for activity. Therefore, the biphasic kinetics may simply be a reflection of the different reaction rates of these residues. (It is interesting to note that extrapolation of the slow and fast slopes in Figure 2 to zero time reveals that 33% of the

activity was lost due to the slow inactivation and 66% due to the rapid inactivation; it is not known how this relates to Nishimura's three important arginine groups.) Since none of the substrates protect selectively or completely against either phase of inactivation (Figure 3) it was felt that the modification of *E. coli* succinyl-CoA synthetase by phenylglyoxal was too complex to allow localization of the binding sites through subunit isolation.

The inactivation of *E. coli* succinyl-CoA synthetase by NBD-Cl showed pseudo-first-order kinetics and Mg^{2+} and ATP- Mg^{2+} afforded partial protection against the inactivation (Figure 4). This was also found to be the case when the enzyme was inactivated by N-hexylmaleimide (76). Figures 5 and 6 show that approximately 3.0 sulfhydryl groups per α, β tetramer are important for activity and that a maximum of 16 sulfhydryl groups per tetramer can be titrated. Previous work with methane thiolyating reagents, 5,5'-dithio-bis(2-nitrobenzoic acid) and permanganate (77,78) has also indicated that of the 16 to 18 titratable sulfhydryl groups, 3.0 to 5.0 appear important for enzyme activity. When the NBD-Cl modified subunits were isolated and refolded with unmodified subunits, no activity was regained. This result demonstrates that the modification of 4.0 sulfhydryl groups in either the α or β subunit is sufficient to inactivate the enzyme as a whole. This is consistent with the hypothesis that a cluster of sulfhydryl groups may be located near or within the active site region

of the enzyme which lies at the point of contact of the α and β subunits (76). Alternatively, these modified groups may be distant from the active site but capable of promoting inhibitory conformational changes throughout the entire refolded enzyme.

III. OXYGEN EXCHANGE AS A PROBE FOR CATALYTIC COOPERATIVITY IN SUCCINYL-COA SYNTHETASE. PART 1: A STUDY ON HYBRID SUCCINYL-COA SYNTHETASE MOLECULES

A. Introduction

Boyer and his co-workers have recently suggested that catalytic cooperativity (as defined in Chapter 1) occurs between the subunits of *E. coli* succinyl-CoA synthetase (29). A simple model illustrating the operation of catalytic cooperativity in succinyl-CoA synthetase is shown in Figure 7. Actually, the more specific alternating sites mechanism has been depicted for the sake of clarity. The phosphoenzyme is pictured as having a configuration characterized by two unequivalent active sites. This concept of phosphorylation resulting in a different conformation is in keeping with the observation that phosphorylation at only one of the two potential active sites in the enzyme results in profound changes in the reactivity of the enzyme to proteases (57). The hatched side of the enzyme molecule is visualized as being in a conformation ready to interact with ATP while the other (phosphorylated) side of the molecule is in a configuration poised to carry out further catalysis, namely production of succinyl-CoA. In the model shown in Figure 7, the reaction of succinate and CoA with E-P gives rise to the production of succinyl-CoA which remains tightly bound to the enzyme. In order to release the succinyl-CoA, it is proposed that ATP must either bind to or phosphorylate the

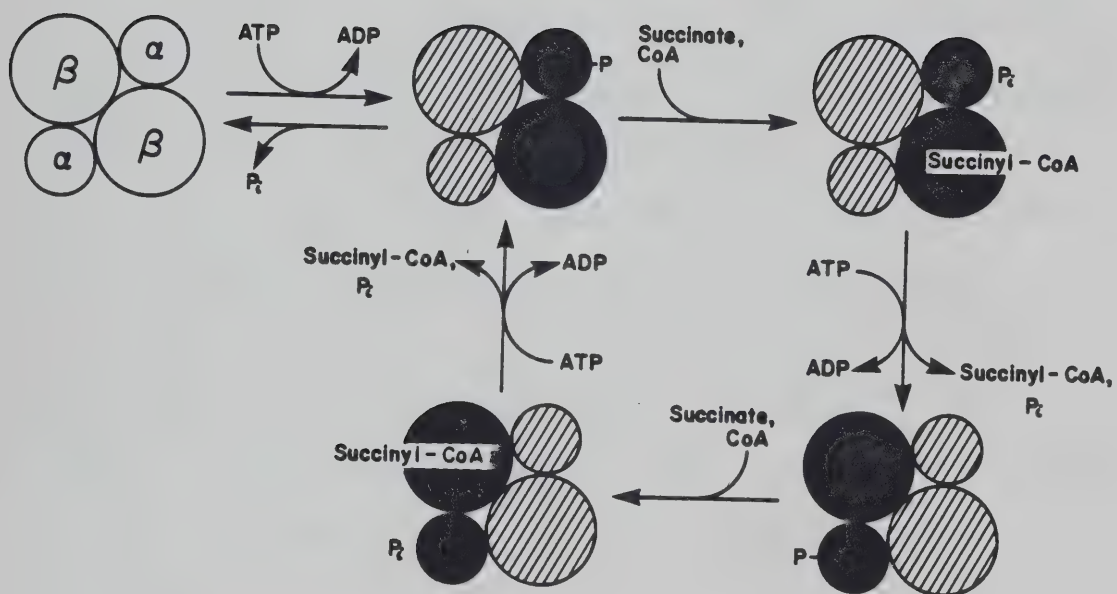


Fig. 7 - Model of catalytic cooperativity in succinyl-CoA synthetase

other (hatched) side of the molecule. This is envisioned as promoting a conformational change throughout the enzyme thereby loosening the binding of succinyl-CoA. In this way, one catalytic cycle is complete and the two halves of the molecule switch conformations. Thus, the subunits may be seen as alternating between the 'catalytic' configuration and the 'ATP binding' configuration. If the release of succinyl-CoA were a rate limiting step, then this mechanism would increase the maximum velocity of the overall reaction by providing for a low binding constant for the product. Although not explicitly indicated in Figure 7, the general model includes the possibility that some other intermediate step and not the release of product may be affected by ATP binding.

The approach developed by Bild, Janson and Boyer for the detection of catalytic cooperativity in succinyl-CoA synthetase involves the measurement of the extent of oxygen exchange between medium [^{18}O]Pi and succinate per molecule of ATP cleaved during steady state succinyl-CoA synthesis. A pronounced increase in the relative rate of oxygen exchange catalyzed by *E. coli* succinyl-CoA synthetase was observed as the ATP concentration was lowered (29). The interpretation of this result as supportive of catalytic cooperativity may be developed from a consideration of the scheme shown in Figure 8. This figure shows the interconversions of bound substrates and intermediates that give rise to oxygen exchange. Enzyme-bound reactants are represented by those

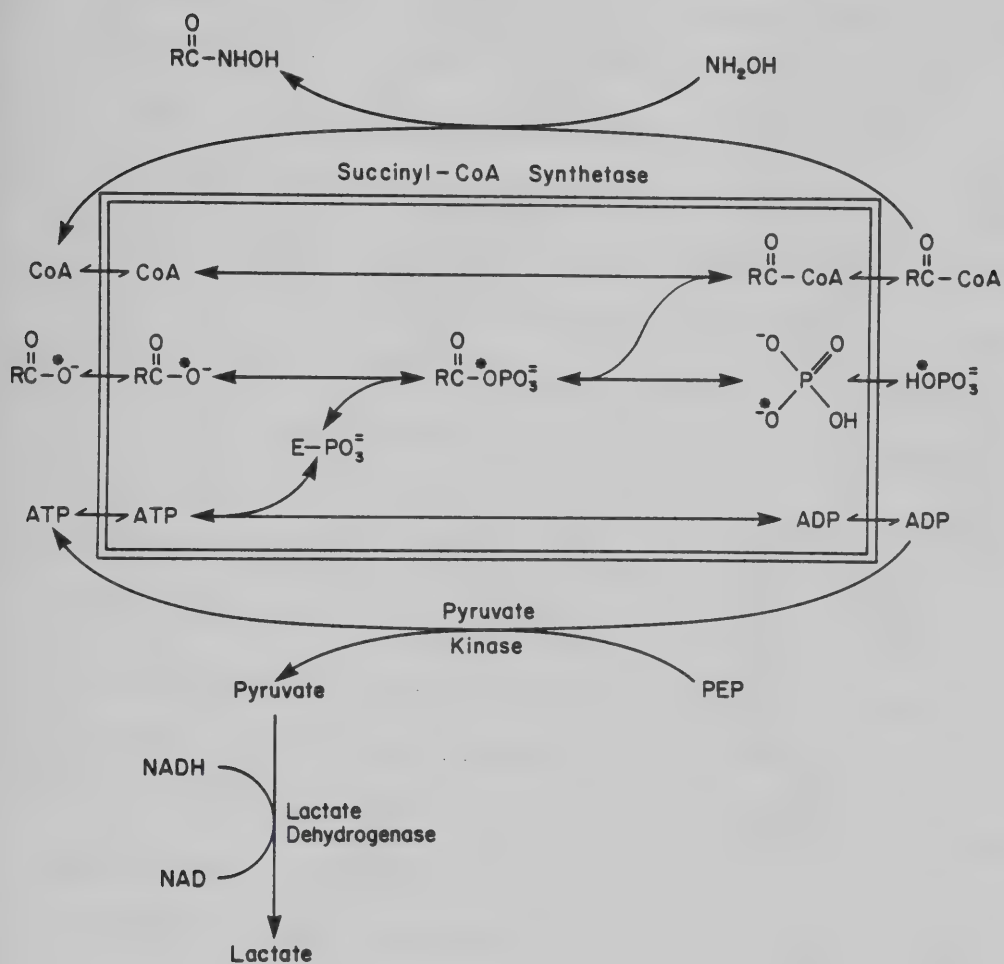


Fig. 8 - Schematic representation of the *E. coli* succinyl-CoA synthetase reaction. $\text{R} = -\text{CH}_2\text{CH}_2\text{COO}^-$

within the box. It can be seen that during net synthesis of succinyl-CoA at least one oxygen from succinate must be transferred to each Pi formed. However, if the inter-conversion of bound reactants occurs prior to release of Pi, more than one oxygen could appear in each Pi released. For oxygen atoms to exchange between medium Pi and medium succinate enzyme-bound phosphate must react with bound succinyl-CoA to form the succinyl phosphate intermediate and then E-P, with subsequent reversal of intermediate reactions including the on-off steps for succinate and Pi. Since the exchange between Pi and succinate was found to be quite rapid in the presence of low levels of succinyl-CoA, hydroxylamine was included to keep the concentration of this product in the medium near zero. ATP levels were kept constant by a pyruvate kinase ATP-regenerating system. The approximate progress of the reaction was monitored by including lactate dehydrogenase and following the oxidation of NADH which is proportional to the net flux of the overall reaction. It was proposed by Bild *et al.* (29) that the observed reduction in the relative rate of exchange at high ATP concentrations results from ATP interaction at one site promoting intermediate steps crucial to oxygen exchange at the other site. For example, if attachment of ATP to one-half of the enzyme molecule decreases the relative residence time or reactivity of bound succinyl-CoA, or increases the rate of formation of the succinyl phosphate intermediate, then the extent of oxygen exchange will be

reduced.

To test the validity of this hypothesis, I have carried out experiments which take advantage of our ability to separate the subunits of *E. coli* succinyl-CoA synthetase and then reconstitute the active enzyme. Using these techniques along with the experimental system developed by Bild *et al.* I have attempted to construct hybrid enzyme molecules containing subunits that are chemically modified and to investigate the oxygen exchange patterns of these hybrids.

B. Experimental Design

Central to the success of this experimental approach is the preparation of a suitable hybrid species of the *E. coli* succinyl-CoA synthetase molecule which has one of its two active sites modified. Since catalytic cooperativity involves interactions between active sites, it was felt that the oxygen exchange kinetics of this one-sided hybrid would test the theory, and would possibly give insight into the details of its operation.

The hybrid enzyme was prepared by inactivating the native enzyme with a sulfhydryl reagent, purifying the modified subunits, and then refolding appropriate mixtures of normal and modified subunits. Since the intact enzyme was modified it was anticipated that surface sulfhydryl groups, and not those protected by subunit contacts, would be affected. Therefore, the modified subunits were expected to retain their ability to refold.

The test for catalytic cooperativity involved the introduction of increasing amounts of modified α subunit (α') into the native tetramer. These preparations, which would contain increasing amounts of one-sided enzyme ($\alpha\alpha'\beta_2$), were examined for their ability to catalyze oxygen exchange. There are three possible outcomes from this experiment. Firstly, the extent of exchange might be insensitive to increasing amounts of α' . This would be expected if catalytic cooperativity were not operating, i.e. if the presence of an inactive α' in one half of the enzyme did not affect the relative amount of exchange catalyzed by the active half of the molecule. Secondly, the amount of oxygen exchange might increase with increasing α' concentrations. This result may be interpreted as being supportive of catalytic cooperativity. That is, if the inactive subunit were unable to interact with ATP it would also be unable to affect intermediate steps in the neighbouring active subunit and the amount of oxygen exchange catalyzed per succinyl-CoA formed would be high, because of the high residence time for succinyl-CoA in the simple model of Figure 7. Finally, an increase in the amount of α' might result in a decrease in the exchange rate. This would occur if α' were 'frozen' in a configuration resembling the phosphorylated state whereby it would be capable of inducing its partner to catalyze a low relative rate of exchange. Therefore this result would also be consistent with the operation of catalytic cooperativity.

C. Experimental Procedures

1. Preparation of the hybrid enzyme

The NBD-Cl modified α subunit (α') was prepared according to the procedures described in the preceding chapter. The protein concentrations of α' and the normal subunits were determined by the protein-dye binding method of Bradford (69) using native succinyl-CoA synthetase as the standard. The hybrid succinyl-CoA synthetase was prepared by essentially the same method as that developed by Pearson (54) for the recombination of α and β subunits into active enzyme, except varying amounts of α' were added. More specifically, equimolar amounts of α and β subunits, both dissolved in 6 M urea, 5% acetic acid, 0.1 mM EDTA, and 0.5 mM dithiothreitol, were mixed so that the resulting protein concentration was 1.2 to 1.3 mg/ml. Ten-microliter aliquots of that solution were added to 35 μ l of the acidic urea solution which contained varying amounts of α' (the concentration of α' was adjusted so that the molar ratio of α'/α ranged from 0 to 5). This 45 μ l sample was neutralized with 67.5 μ l of 1 M Tris, pH 8.7 and then rapidly diluted into 250 μ l of a solution containing 50 mM Tris-HCl (pH 7.4), 0.4 mM ATP, 10 mM MgCl_2 , 10 mM succinate, and 0.1 mM dithiothreitol. After incubation of the mixture at 26° for 1 to 3 hours, 200 μ l aliquots of the refolded hybrid proteins were assayed for enzyme activity and ability to catalyze oxygen exchange.

2. Preparation of [^{18}O]Pi

Highly enriched Pi was made by the hydrolysis of PCl_5 in enriched water. This method was developed by Risley and Van Etten (79) and modified by Hackney *et al.* (80). I followed essentially the same procedure as that used by Hackney *et al.* but an excess of enriched water was not used. Accordingly, the following changes were made: 1 ml (instead of 4 ml) of enriched water was reacted with the PCl_5 , this mixture was neutralized with 2.4 g of imidazole (instead of 5 g) and then it was diluted to 200 ml (not 4 liters) before loading on the column. Otherwise, the procedures were identical. Two preparations were made, the first was 90% enriched while the second was 97% ^{18}O .

3. The reaction mixture

The reaction mixture in which the oxygen exchange was carried out was essentially the same as that developed by Bild *et al.* (29). The exact conditions used were as follows: the mixture contained, at pH 7.2, 60 mM Hepes-NaOH, 10 mM MgCl_2 , 20 mM disodium succinate, 0.20 mM CoA, 5 mM [^{18}O]Pi, 1.0 mM phosphoenol pyruvate, 480 mM hydroxylamine hydrochloride, 0.40 mM NADH, 40 units of pyruvate kinase, 110 units of lactate dehydrogenase, 150 μM ATP, and a 200 μl aliquot of the hybrid enzyme solution containing approximately 7 μg of refolded α and β plus varying amounts of α' . Reactions were run at 25°C in a total volume of 1.5 ml. The stock solutions of ATP, CoA and NADH were prepared fresh each day. The solution containing pyruvate kinase and

lactate dehydrogenase was dialyzed against the Hepes buffer prior to use to remove ammonium sulfate. The hydroxylamine hydrochloride was neutralized to pH 7.0 with concentrated KOH.

The approximate progress of the reaction was monitored by following the decrease in absorbance at 340 nm accompanying the oxidation of NADH. The blank cell contained the same reaction mixture minus the enzyme. After starting the reaction by the addition of enzyme, it was allowed to proceed until approximately 200 nmoles of product were formed (an A_{340} change of 0.8-0.9). Since this assay was found to be unreliable, especially when the reaction times were several hours, the concentration of succinohydroxamic acid was determined more accurately by estimating the succinohydroxamate as its Fe^{3+} complex according to the method of Kaufman *et al.* (81). This was done by quenching 0.5 ml of the reaction mixture in 0.5 ml of a solution containing 5% $FeCl_3$ -3N HCl-12% TCA. The mixture was mixed for 1 minute and then the precipitated protein was removed by centrifugation for 1 minute in an Eppendorf 5412 centrifuge at 15,000 rpm. The absorbance at 540 nm of the supernatant solution was determined immediately using 0.5 ml water plus 0.5 ml $FeCl_3$ -HCl-TCA as a blank. A standard curve was made using succinohydroxamic acid prepared from succinic anhydride by the method of Lipmann and Tuttle (82).

4. Analysis of [^{18}O]Pi by ^{31}P NMR

Recently, Cohn and Hu (83) have demonstrated that small chemical shifts in the ^{31}P NMR of phosphate compounds are induced by substitution of ^{18}O for ^{16}O . I used this method as an alternative to the mass spectroscopic techniques used by Boyer and co-workers for the determination of the distribution of ^{18}O -containing phosphate species. The ^{31}P NMR method was chosen since it involves minimal sample preparation and a high frequency NMR spectrometer was available.

The samples were prepared for NMR analysis as follows: At the same time that the 0.5 ml aliquot of the reaction mixture was stopped for analysis of product, the remaining 1 ml was quenched by adding it to 200 μl of chloroform. The solution was mixed for 1 minute and frozen in a dry ice-ethanol bath until all the incubations were complete. The sample was then thawed and the chloroform layer centrifuged out. To the supernatant, D_2O (700 μl) and EDTA (200 μl of a 200 mM solution) were added; the pH was adjusted to 8.3 with 1 N NaOH and the solution (approximately 2 ml) was transferred to a precision NMR tube (Wilmad). A teflon vortex suppressor was placed in the tube.

The ^{31}P NMR spectra were recorded on a Bruker HDX-270 spectrometer at 109.29 MHz. They were done in the Fourier Transform Mode using a 22 μsec pulse (32 μsec = 90° flip angle) and an acquisition time of 6.8 sec. The spectral width was 100 Hz and a linebroadening of 0.10 Hz was

introduced by computer digital filtering. The number of scans was usually 200.

The percentage distribution of the five species of inorganic phosphate, i.e., $P^1'O_4$, $P^1'O_3$, $^{18}O_1$, $P^1'O_2, ^{18}O_2$, $P^1'O, ^{18}O_3$, $P^{18}O_4$ was quantitated by integrating the individual peaks using a standard Nicolet 1180 program. The separation between the peaks at 109.29 MHz was 2 Hz (0.02 ppm) and all peaks were well resolved. (Sample spectra are shown in the following chapter, Figure 12). The error, as estimated from the signal to noise ratios in the spectra, was less than 3% for measurements of the larger peaks but could be as large as 50% for the smaller peaks. This results in a maximum error of between 2 and 3% in the measurement of the average ^{18}O content of the Pi.

5. Sources of Chemicals

ATP was obtained from Terochem (Edmonton, Canada), Coenzyme A was from P.L. Biochemicals or Boehringer Mannheim, phosphoenolpyruvate, HEPES, and NADH were purchased from Sigma, Tris (ultra pure) from Schwarzmann, hydroxylamine hydrochloride from J.T. Baker, $H_2, ^{18}O$ from Merck, Sharp and Dohme, D_2O from Bio-Rad and PCl_5 from Fisher. Pyruvate kinase and lactate dehydrogenase were purchased from Sigma and were Type II from rabbit muscle. The pyruvate kinase had a specific activity of approximately 450 units/mg while the lactate dehydrogenase had 900 units/mg. Other chemicals were analytical grade.

D. Calculations

1. *Specific activity of succinyl-CoA synthetase*

One unit is defined as the amount of enzyme catalyzing the formation of 1 micromole of product in 1 minute at 25°C. Specific activity is expressed as units per milligram of protein. The activity of the enzyme was calculated from the NADH assay by measuring the change in absorbance at 340 nm per minute and using a molar extinction coefficient for NADH of 6.22×10^3 (84). To calculate the activity from the succinohydroxamate assay the duration of the incubation was recorded and the amount of product formed during this time was estimated using Kaufman's assay (81) and the succinohydroxamate standard curve. In general, the enzyme activities calculated from the two assays were consistent except for long incubation times when the NADH assay underestimated the amount of product formed.

2. *The amount of oxygen exchange*

The total gram-atoms of oxygen exchanged between medium [^{18}O]Pi and succinate was calculated from the equation used by Bild *et al.* (29):

micromoles of oxygen exchange = $[-XY/(X+Y)]\ln(1-F)$ where F = the fraction of isotopic equilibrium and X and Y = the amount of exchanging components in micromoles.

Example calculation:

Reaction volume = 1.5 ml

Concentration of succinate = 20 mM

Concentration of [^{18}O]Pi = 5 mM

Original enrichment of [^{18}O]Pi = 90% = c

Enrichment of [^{18}O]Pi after exchange = 74% = y

Pi contains 4 equivalent oxygens; therefore Pi contributes a $4 \times 5 \text{ mM} = 20 \text{ mM}$ pool of oxygen.

Succinate contains 4 equivalent oxygens; therefore succinate contributes a $4 \times 20 \text{ mM} = 80 \text{ mM}$ pool of oxygen.

At isotopic equilibrium then, the atom % ^{18}O in Pi = $[20/(20+80)]c = 0.2c = 18\%$

Therefore, the fraction of isotopic equilibrium reached in this case = $F = c - y / c - 0.2c = 90 - 74 / 90 - 18 = 0.22$

X = micromoles of exchanging oxygen contributed by succinate
 $= 80 \text{ micromole/ml} \times 1.5 \text{ ml} = 120 \text{ micromoles}$

Y = micromoles of exchanging oxygen contributed by Pi = 20
 $\text{micromole/ml} \times 1.5 \text{ ml} = 30 \text{ micromoles.}$

$[-XY/(X+Y)] = -22 \text{ micromoles}$

Therefore the micromoles of oxygen exchange
 $= [-22] \ln(1 - 0.22) = 5.5.$

This quantity was then divided by the amount of product

formed (as estimated from the succinohydroxamate assay) to give the relative amount of oxygen exchange.

E. Results

1. *Specific activity of the hybrid enzymes*

The extent of inactivation of the hybrid resulting from addition of α' to the refolding $\alpha_2\beta_2$ enzyme is shown in Figure 9. The specific activity was determined using the succinohydroxamate assay and was calculated in terms of the amount of $\alpha_2\beta_2$ present. Also shown is the theoretical activity loss calculated for three model systems. An understanding of these calculations may be developed by consideration of Figure 10. If α' and α refold into the $\alpha_2\beta_2$ enzyme molecule at the same rate then the actual composition and proportion of molecular species in solution will follow a binomial distribution as represented in Figure 10. For example, when equal amounts of α and α' are added to the refolding solution, one-fourth of the enzyme molecules will contain no modified α subunits (E_1), one-half will have one modified α ($E_2 + E_3$), and one-fourth will have both α subunits modified (E_4).

The composite activity of this hybrid enzyme mixture will depend on the catalytic roles of the two α subunits in the $\alpha_2\beta_2$ tetramer.

Model 1: If both α subunits are essential for activity, only molecules of type E_1 will be active. Hence, when the ratio of α to α' is unity, a 75% loss of enzyme activity will be

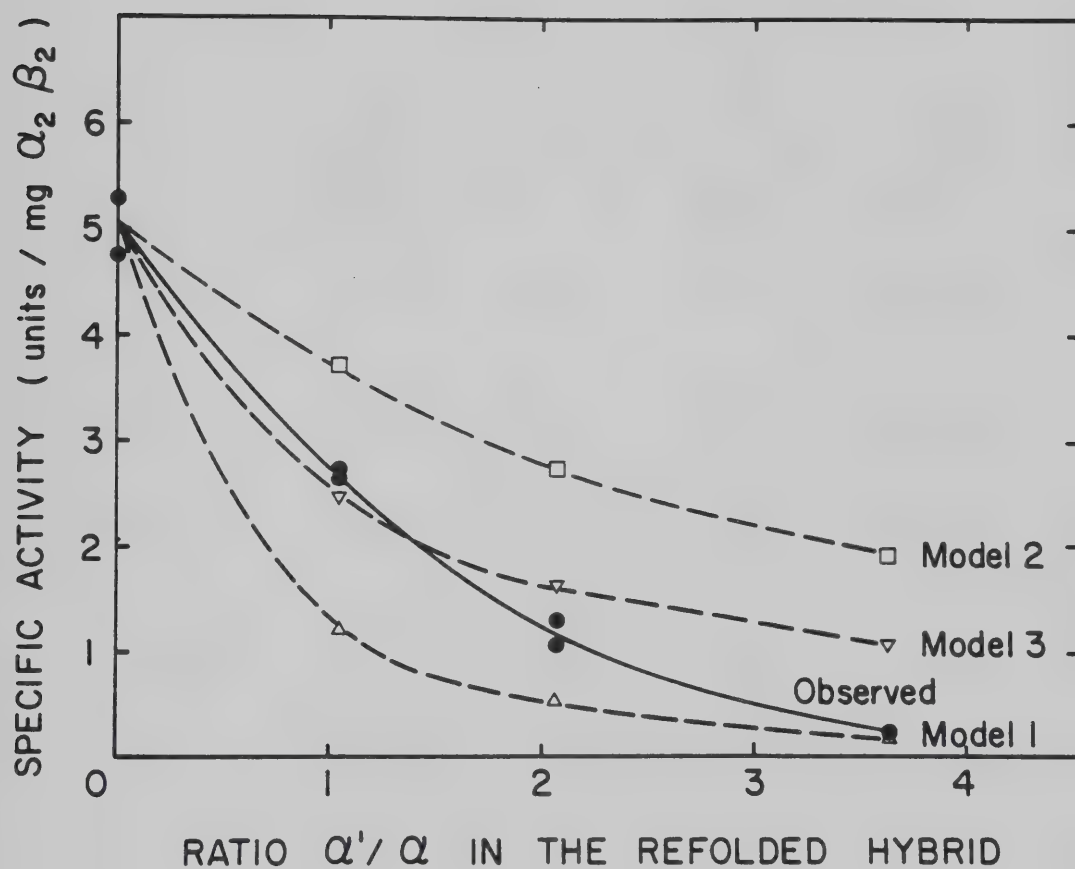






Fig. 9 - Observed and theoretical specific activities of the hybrid enzymes.

	E_1	E_2	E_3	E_4
				
Model 1:	active	inactive	inactive	inactive
Model 2:	active	active	active	inactive
Model 3:	active	$\frac{1}{2}$ active	$\frac{1}{2}$ active	inactive
Distribution:	a^2	ab	ba	b^2

where a = the relative amounts of α and b = the
the relative amount of α' .

Figure 10 - Molecular species of hybrid enzyme.

observed.

Model 2: If one α subunit can substitute for the other, i.e. if both subunits must be modified for inactivation to occur, types E_1 , E_2 and E_3 will be active and only 25% loss of activity will be observed.

Model 3: If the two α subunits act independently of each other during catalysis, then the enzyme molecules containing one modified α (E_2 and E_3) should be one-half as active as the unmodified molecule and the loss of activity will be 50%.

It can be seen from Figure 9 that the observed loss of activity was different from that calculated for any of the three model systems.

2. The oxygen exchange kinetics of the hybrid enzymes

The effect of increasing the proportion of α' in the hybrid enzyme mixture on the relative amount of exchange catalyzed by the hybrid preparation is shown in Figure 11. Three separate experiments were done using different preparations of α , β and α' . The first of these showed a general decrease in the amount of exchange as α' was added (this preliminary result was reported in ref. 85), the trend in the second experiment was towards an increase in the amount of exchange and in the final experiment there was initially an increase and then a decrease in the exchange as α' was added. In view of the variability of these results, it seemed appropriate to estimate the amount of error that can be expected in this type of experiment.

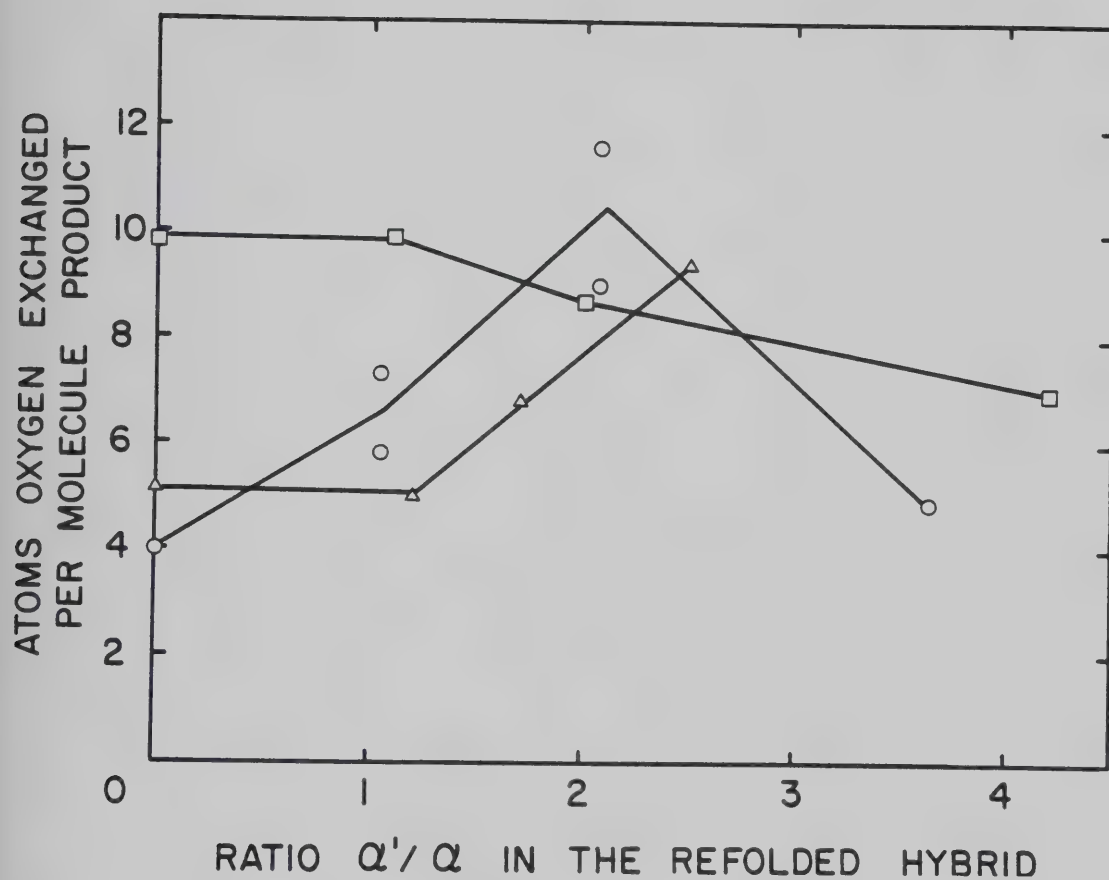


Figure 11 - Amount of oxygen exchange catalyzed by the hybrid enzymes.

3. Estimation of error

Since the relative amount of oxygen exchange is a prescribed function of a set of measured numbers and their errors, the propagation of errors can be estimated according to the equations described in ref. (86). This derivation is shown below:

micromoles of oxygen exchange $= [-XY/(X+Y)] \ln(1-F)$ where F = the fraction of isotopic equilibrium and X and Y = the amount of exchanging components in micromoles.

As shown in the section on calculations, when the reaction volume = 1.5 ml, the concentration of succinate = 20 mM and the concentration of P_i = 5 mM, the equation reduces to the following form:

micromoles of oxygen exchange $= [-22] \ln[1 - (c - y/c - 0.2c)]$

where c = original enrichment of [^{18}O] P_i and y = enrichment of [^{18}O] P_i after exchange. It follows that:

$$O = \frac{\text{atoms oxygen exchanged}}{\text{molecule product}} = \frac{[-22 \times 10^3] \ln[1 - (c - y/c - 0.2c)]}{P}$$

where P = amount of product formed in nmoles. Using the methods in ref. (86) the error in O will be:

$$\Delta O^2 = \left(\frac{\partial O}{\partial c} \times \Delta c \right)^2 + \left(\frac{\partial O}{\partial y} \times \Delta y \right)^2 + \left(\frac{\partial O}{\partial P} \times \Delta P \right)^2$$

$$\text{where } \frac{\partial O}{\partial c} = \frac{[22 \times 10^3]}{Pc[y - 0.2c]} y$$

$$\frac{\partial O}{\partial x} = \frac{[-22 \times 10^3]}{P[y - 0.2c]}$$

$$\frac{\partial O}{\partial P} = \frac{[22 \times 10^3]}{P^2} \ln [(y - 0.2c)/0.8c]$$

The maximum error in c and y (Δc and Δy) as estimated from the signal to noise ratios in the ^{31}P NMR spectra is approximately 3.0%. The maximum error expected for the measurement of P (ΔP) was estimated from the succinohydroxamate standard curves and is approximately 2 nmoles of product. Using a specific example from the hybrid enzyme experiments, the maximum error expected in O can be calculated:

$$c = 90\% \pm 3.0\%$$

$$y = 81\% \pm 3.0\%$$

$$P = 250 \text{ nmoles} \pm 2 \text{ nmoles}$$

$$\frac{\partial O}{\partial c} = \frac{(22 \times 10^3)(81)}{(250)(90)[81 - 0.2(90)]} = 1.3$$

$$\frac{\partial O}{\partial y} = \frac{(-22 \times 10^3)}{(250)[81 - 0.2(90)]} = -1.4$$

$$\frac{\partial O}{\partial P} = \frac{(22 \times 10^3) \ln [77 - 0.2(90)/(0.8)(90)]}{(250)^2} = -0.07$$

$$\text{Therefore } O = \sqrt{(1.3 \times 3.0)^2 + (-1.4 \times 3.0)^2 + (-0.07 \times 2.0)^2} = 5.7.$$

This error does not take into account the variations in the hybrid enzyme preparations or the reaction mixtures. However, it is still large enough to account for most of the variability seen in the oxygen exchange data of Figure 11.

F. Discussion

There are several possible explanations for the finding that the observed loss of activity due to the addition of α' does not coincide with the loss predicted for three models of inactivation (Figure 9). First of all, this result may be interpreted as being supportive of catalytic cooperativity. That is, if the function of one α subunit in the tetramer is to make its partner a better catalyst, then the modifications of that subunit may only partially inactivate its partner. This would lead to a more complex pattern of inactivation than expected for any of the three models.

However, there are other rationalizations for the observed inactivation. For example, it was assumed that α' and α refolded into the tetramer at the same rate. If this were not so, then the activation by α' would differ from that calculated for the model systems. Also, there have been reports in the literature that the NBD modification can be transferred from tyrosine to an amino group in lysozyme (87) and in the mitochondrial ATPase (88), and in model systems transfer from -SH groups to nitrogen has been demonstrated (72). These results suggest that, if a suitably orientated amino group were present, an intramolecular transfer of the NBD modification from -SH groups to basic amino acid side-chains could occur in the refolding succinyl-CoA synthetase molecule. If this transfer takes place the inactivation would again be expected to be more complex than predicted.

Within the substantial limits of experimental error, the data of Figure 11 suggest that the oxygen exchange per ATP cleaved does not change significantly as the proportion of α' is increased in the hybrid enzyme preparation. This result may be interpreted as being unsupportive of catalytic cooperativity (as discussed under the section on experimental design). The validity of this conclusion was further investigated by experiments done to confirm that the refolded enzyme (no α' added) catalyzed an increase in the relative rate of oxygen exchange as the ATP concentration was lowered (i.e. the refolded enzyme should exhibit the same exchange kinetics as native *E. coli* succinyl-CoA synthetase). It was found that the exchange catalyzed by the refolded enzyme remained low and was not only insensitive to the presence of α' but also to the concentration of ATP. The source of this anomaly was eventually traced to the fact that the refolded enzyme was assayed at a much lower protein concentration than the native enzyme. It was discovered that the exchange catalyzed by the native enzyme was also insensitive to ATP when the enzyme was assayed at a sufficiently low concentration. This enzyme concentration effect is investigated in detail in the next chapter.

I did not pursue the hybrid enzyme experiments further for several reasons. First of all, it would be technically difficult to raise the concentration of the hybrid enzyme to the point where the relative amount of oxygen exchange is sensitive to ATP (and possibly α'). Secondly, as mentioned

above, there exists a possibility that the modification in α' transfers between residues in the refolded enzyme. The absorbance spectrum of the refolded hybrid enzyme ($\alpha':\alpha=1$) showed a diminished peak at 420 nm (this peak being characteristic of the NBD-SH modification) indicating that a transfer may have occurred. If the NBD-residue has moved between subunits, then the hybrid enzyme may not be truly 'one-sited'. Also, as discussed in the next several chapters, *E. coli* succinyl-CoA synthetase appears to exist in a dimer-tetramer equilibrium. For unequivocal interpretation of the oxygen exchange kinetics of the hybrid enzymes, it would be necessary to show that the equilibrium was not perturbed by the presence of α' .

IV. OXYGEN EXCHANGE AS A PROBE FOR CATALYTIC COOPERATIVITY IN SUCCINYL-COA SYNTHETASE. PART 2: THE EFFECT OF ENZYME CONCENTRATION

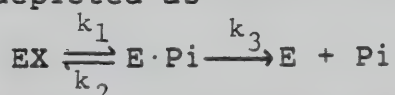
A. Introduction

The hybrid enzyme experiments described in the preceding chapter led to the discovery that the oxygen exchange catalyzed by *E. coli* succinyl-CoA synthetase is modulated not only by ATP concentration as previously reported (29) but also by enzyme concentration. This chapter involves a detailed examination of this phenomenon for both the *E. coli* and pig heart enzyme.

The dependence of a kinetic parameter (in this case the amount of oxygen exchange) on the protein concentration used in the experiment may indicate that the enzyme is undergoing a self association-dissociation reaction. Previous sedimentation equilibrium studies on *E. coli* succinyl-CoA synthetase have shown that there is a marked dependence of the molecular weight upon the enzyme concentration (48). At high concentrations the enzyme exists predominantly as an $\alpha_2\beta_2$ tetramer whereas at low concentrations some of the enzyme appears to dissociate to an $\alpha\beta$ dimer. This observation lends support to the concept that the enzyme concentration dependence of the oxygen exchange reaction may be a manifestation of a dimer-tetramer equilibrium of the enzyme in the reaction mixture.

However, Bild *et al.* (29), in considering their analysis of the distribution of the five ^{18}O -labelled Pi species formed during the exchange reaction, argued that this kind of enzyme heterogeneity was unlikely, concluding that the ^{18}O -patterns seen in their experiments were indicative of catalysis by a single catalytic route.

This chapter describes application of the same theoretical analysis on the observed ^{18}O distributions in Pi in order to re-evaluate possible contributions of two catalytic routes (one route for the dimer and one for the tetramer) under our specific experimental conditions. Such analysis of the medium succinate-Pi exchange involves the estimation of the partition coefficient, Pc. In general, Pc represents the probability (0 to 1.0) of an enzyme-bound Pi molecule undergoing an oxygen exchange step. For the case of succinyl-CoA synthetase, the exchange and release steps may be depicted as



where EX represents the succinyl phosphate intermediate. The partition coefficient is equal to $k_2/(k_2 + k_3)$ and is equivalent to the effective likelihood that a bound Pi is transiently converted back to bound succinyl phosphate during its residence time on the enzyme. If Pc is close to zero ($k_3 \gg k_2$) then only one oxygen atom could be exchanged per encounter of Pi (i.e. the one oxygen atom that is stoichiometrically transferred) and the initially random distribution of ^{18}O in the Pi population will be maintained

during the exchange reaction. If P_c is close to unity ($k_3 < k_2$) then each time P_i binds to the enzyme it will undergo many reversals before its release and will exchange all four of its ^{18}O oxygens with succinate. This would result in the $P^{18}O_4$ species being replaced by the $P^{18}O_4$ species, with no accumulation of partially-labelled species. For intermediate values of P_c the cascade of enriched P_i species is more complex but the complete theoretical treatment of this situation has been described by Hackney (89). A copy of his computer program for performing these calculations was obtained and the latter part of this chapter involves a comparison of the observed and predicted distributions of enriched P_i species. This analysis was done to test whether the exchange process could be adequately described by a single catalytic route with a single value of P_c , or whether two P_c 's characteristic of the dimer and tetramer were evident. The sensitivity and limitations of this approach are discussed.

B. Materials and Methods

The reaction mixture in which the oxygen exchange was carried out in these experiments was essentially the same as that described in the preceding chapter. However, the aliquot of hybrid enzyme was replaced by an aliquot of native *E. coli* or pig heart succinyl-CoA synthetase and ATP was replaced by GTP in the pig heart enzyme experiments. The enzyme and nucleotide concentrations were varied as

described in the text. Other procedures were the same as in the preceding chapter; i.e., the progress of the reaction was monitored using the NADH assay, the amount of product succinohydroxamate was estimated as its Fe^{3+} complex and the ^{18}O distribution in the Pi was analyzed using ^{31}P -NMR. Sample spectra are shown in Figure 12. In this particular experiment, the amount of exchange was varied by changing the concentration of *E. coli* succinyl-CoA synthetase used in the reaction mixture. However, as will be shown below, the same set of spectra can be obtained by varying the pig heart enzyme concentration, by changing the incubation period, or by varying the concentration of nucleotide present.

C. Results

1. Effect of *E. coli* succinyl-CoA synthetase concentration on the oxygen exchange pattern

When Bild *et al.* (29) measured the effect of ATP on the relative extent of oxygen exchange, the ^{18}O loss per ATP cleaved increased approximately 8-fold as ATP was lowered from 150 μM to 3.6 μM (Table 1 in their paper). Figure 13 shows that a similar pattern was obtained when I repeated the experiment using the same concentration of *E. coli* enzyme in the reaction mixture (0.40 μM or 50 $\mu\text{g/ml}$). However, when the enzyme concentration was lowered to 8.5 $\mu\text{g/ml}$ the ATP effect was reduced. There was almost no modulation of exchange by ATP when the enzyme concentration was as low as 4.3 $\mu\text{g/ml}$.

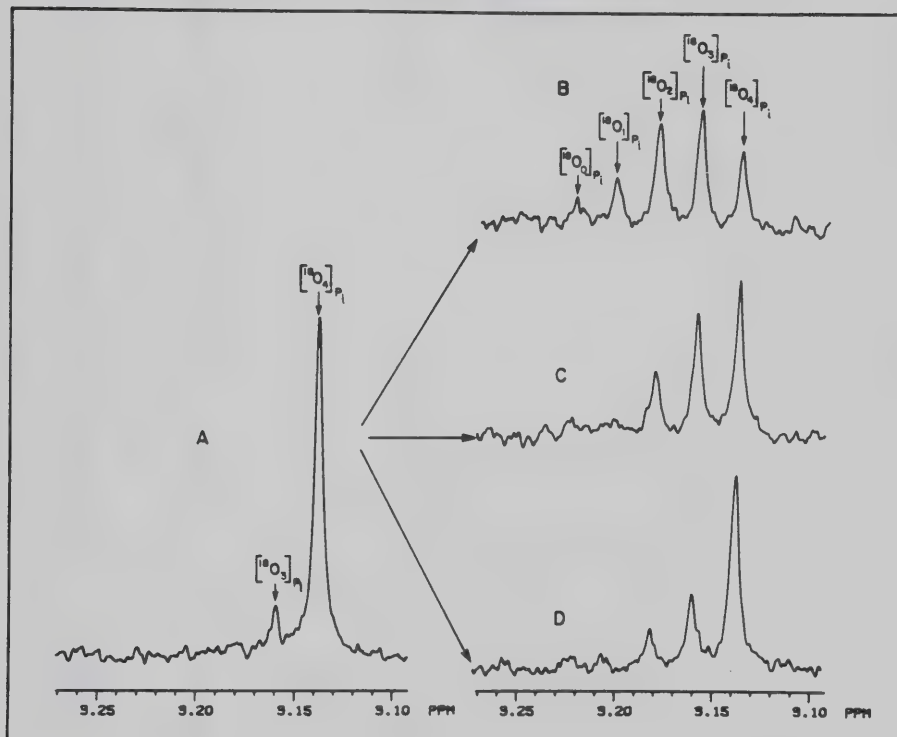
MEASUREMENT OF OXYGEN EXCHANGE USING ^{31}P NMR

Fig. 12 - Sample spectra of exchanged phosphate. A (97% ^{18}O); the original ^{18}O -enriched phosphate sample. B (61.0% ^{18}O), C (70.9% ^{18}O) and D (81.1% ^{18}O); the phosphate after being exchanged in the presence of 37, 12 and 3.5 $\mu\text{g/ml}$ of *E. coli* succinyl-CoA synthetase. The ATP concentration in the reaction mixtures was 5 μM .

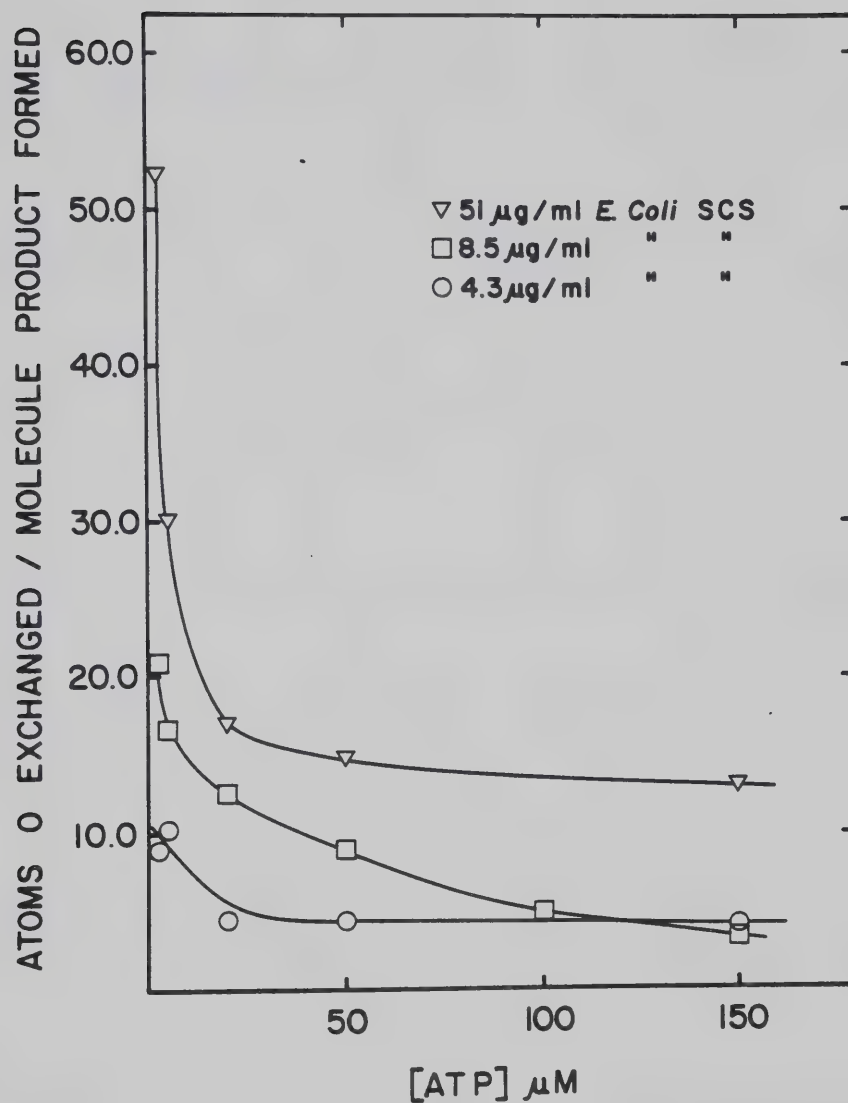


Fig. 13 - Effect of ATP concentration on the relative amount of oxygen exchange at three different *E. coli* succinyl-CoA synthetase concentrations. Reaction conditions are as shown (SCS=succinyl-CoA synthetase).

2. Effect of *E. coli* succinyl-CoA synthetase concentration on the oxygen exchange at a fixed ATP concentration

The results of the previous section show that the maximum enzyme concentration effect can be observed when the ATP concentration is low. This effect was investigated in more detail by varying the concentration of *E. coli* succinyl-CoA synthetase in the reaction mixture from 86 $\mu\text{g/ml}$ to 3 $\mu\text{g/ml}$ while keeping the ATP concentration constant at 5 μM (Figure 14). It can be seen that the ^{18}O loss per ATP cleaved decreases linearly (within experimental error) as the enzyme concentration is lowered.

3. Effect of pig heart succinyl-CoA synthetase concentration on the oxygen exchange pattern

Under the conditions used by Bild *et al.* (29) GTP did not modulate the amount of oxygen exchange catalyzed by pig heart succinyl-CoA synthetase. This experimental finding was in contrast to the result they obtained for the *E. coli* enzyme and, since the pig heart enzyme was considered to be an $\alpha\beta$ dimer, was central to their proposal that the tetrameric *E. coli* enzyme exhibits catalytic cooperativity. However the results presented in Figure 13 show that, when the *E. coli* enzyme concentration is low enough, its oxygen exchange pattern resembles that of the pig heart enzyme; i.e. exchange is insensitive to nucleotide concentration. It seemed logical to test whether the pig heart enzyme would catalyze a nucleotide-sensitive exchange pattern when its concentration was increased. The results are shown in

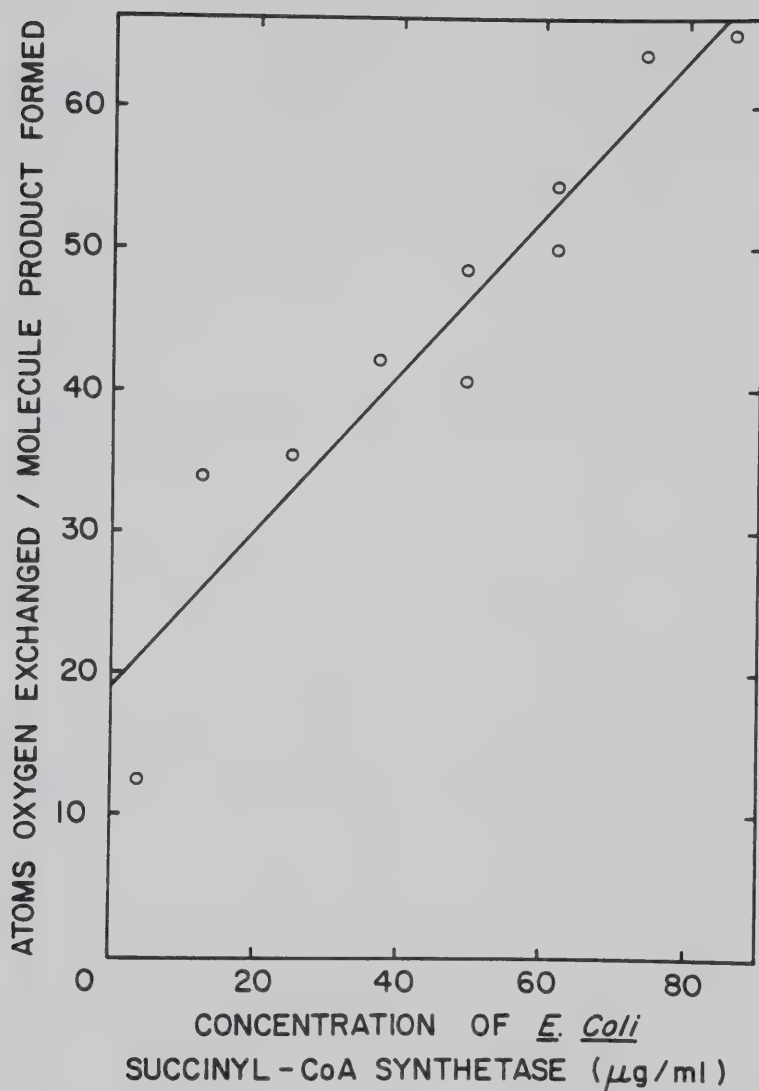


Fig. 14 - Effect of *E. coli* succinyl-CoA synthetase concentration on the relative amount of oxygen exchange at an ATP concentration of 5 μM Reaction conditions are as described in the text.

Figure 15. It can be seen that in our hands also the pig heart enzyme does catalyze a nucleotide insensitive pattern when its concentration is close to that used by Bild *et al.* ($0.40 \mu\text{M}$ or $30 \mu\text{g/ml}$). However, when the enzyme concentration is increased 10-fold the exchange becomes sensitive to nucleotide. Therefore, not only can the *E. coli* enzyme be made to catalyze an exchange pattern that has been thought to be characteristic of the pig heart enzyme, but the pig heart enzyme can also be induced to catalyze an oxygen exchange pattern that is modulated by GTP.

4. Effect of pig heart succinyl-CoA synthetase concentration on the oxygen exchange at a fixed GTP concentration

An experiment analogous to that described above for the *E. coli* enzyme (Section 2) was carried out on the pig heart enzyme. Specifically, the pig heart enzyme concentration was varied from $10 \mu\text{g/ml}$ to $386 \mu\text{g/ml}$ while keeping the GTP concentration constant at $2.5 \mu\text{M}$ (Figure 16). As was the case with the *E. coli* enzyme, the ^{18}O loss per ATP cleaved decreases linearly as the concentration of succinyl-CoA synthetase is lowered.

5. The Partition Coefficient

The evaluation of the partition coefficient (P_c) of bound P_i during succinyl-CoA synthetase catalyzed medium P_i -succinate exchange is reported here. The ratio, R_4 , of the rate of loss of the $\text{P}'^{18}\text{O}_4$ species to the rate of loss of the average ^{18}O enrichment can be used for quantitative evaluation of P_c . R_4 can be related to P_c by

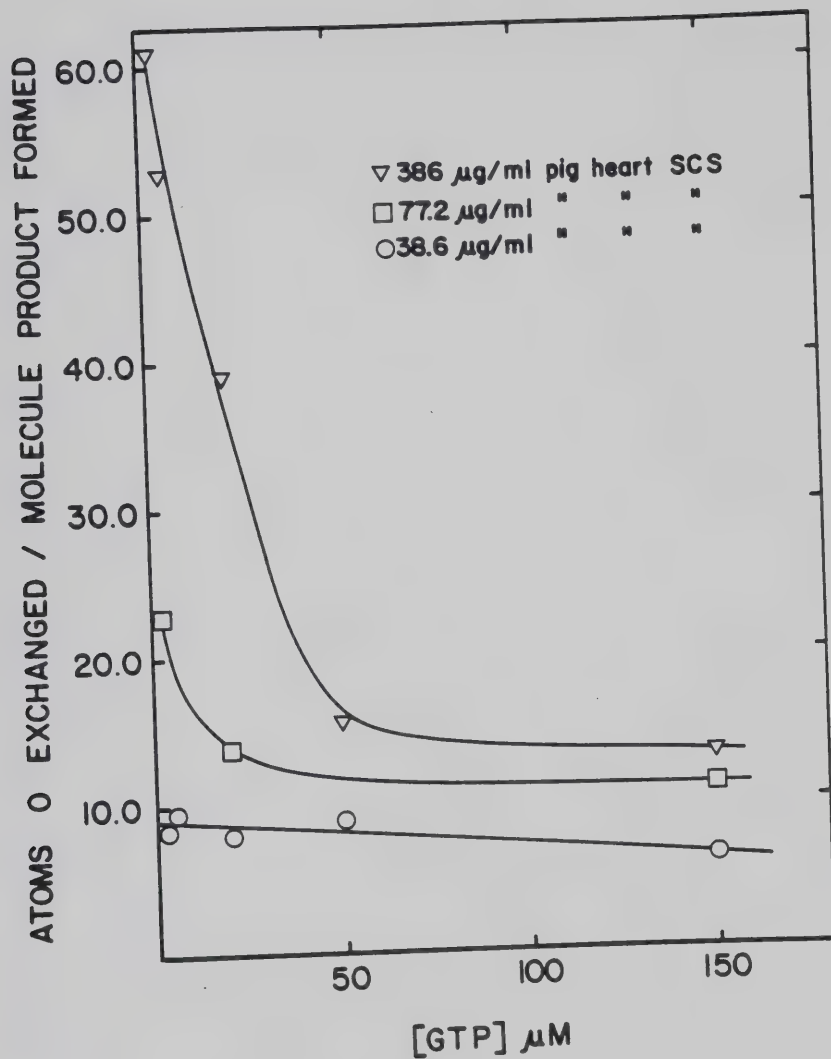


Fig. 15 - Effect of GTP concentration on the relative amount of oxygen exchange at three different pig heart succinyl-CoA synthetase concentrations. Reaction conditions are as shown (SCS=succinyl-CoA synthetase).

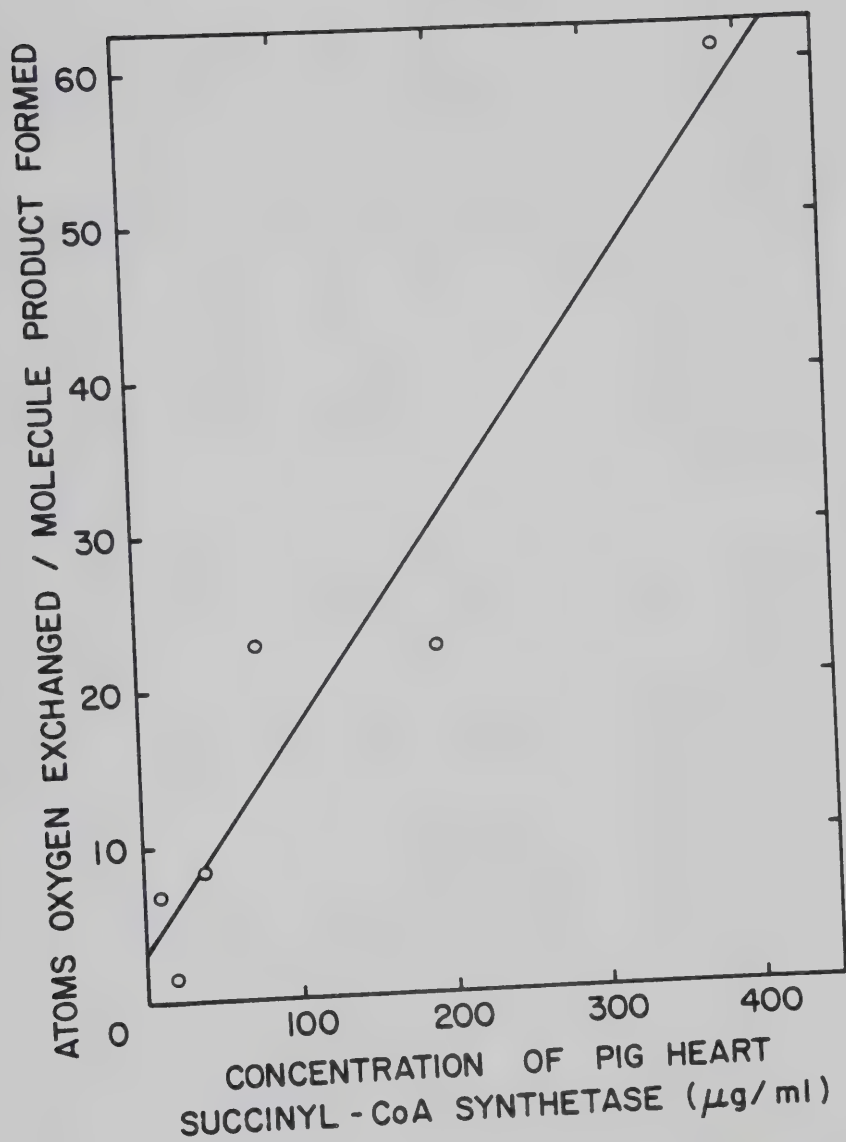


Fig. 16 - Effect of pig heart succinyl-CoA synthetase concentration on the relative amount of oxygen exchange at a GTP concentration of $2.5 \mu\text{M}$. Reaction conditions are as described in the text.

$$P_c = 4 - R_4/3 \quad (90)$$

Figure 17 shows the results of an experiment where the *E. coli* enzyme was allowed to catalyze exchange for increasing time periods before being quenched. It can be seen that the loss of the average ^{18}O enrichment and of the P^{18}O_4 species occurs in a first order process. The ratio of the slope of the two lines (R_4) was calculated to be 3.26, which corresponds to a P_c value of 0.25. This agrees fairly well with the reported range of 0 to 0.2 for the P_c of the succinyl-CoA synthetase exchange reaction (29). This same calculation was done for all the experimental points shown in Figures 13, 14, 15 and 16. (The rates of loss were estimated in these cases from only the initial and final ^{18}O distributions). The calculated P_c value remained relatively constant under all the experimental conditions examined and was consistently between 0.2 and 0.4 for both the pig heart and *E. coli* enzyme. The average of all the determinations was 0.33. It is apparent that P_c is not modulated by changes in either the enzyme or nucleotide concentration used in the reaction mixture.

The above procedure yields an evaluation of P_c but it provides no means of establishing whether the exchange reaction involves a single catalytic route or whether it must be characterized by two or more routes with different values for P_c . The critical test for this involves a comparison of the predicted distribution of all five P_i species with the observed distribution. Theoretical

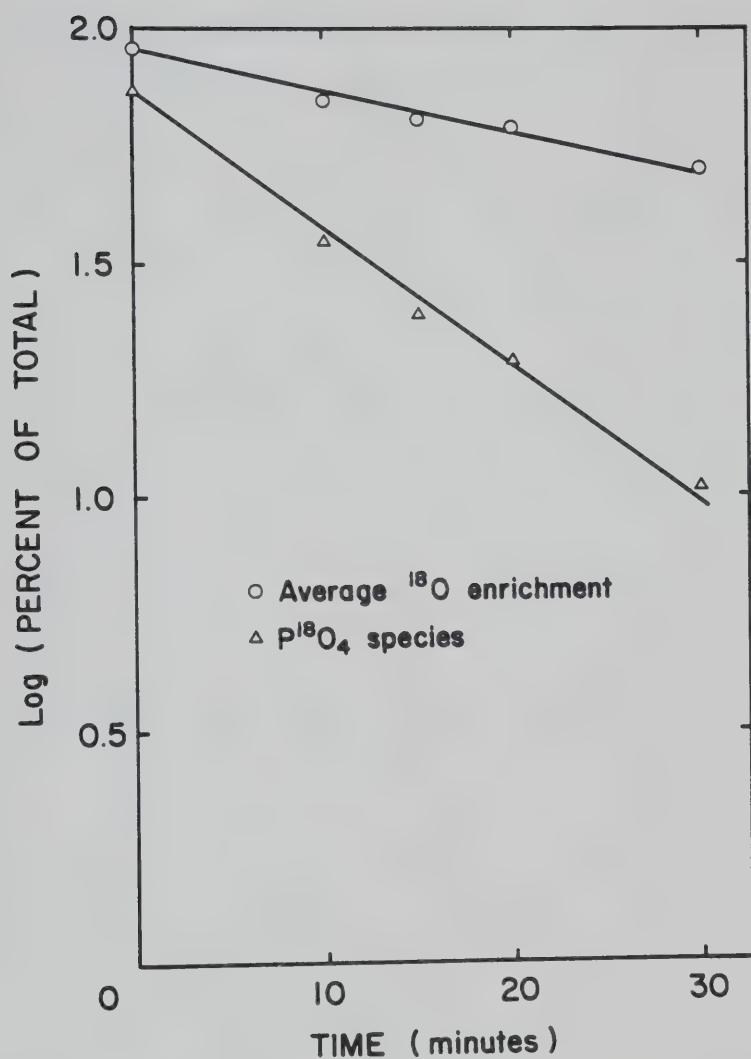


Fig. 17 - Time course for loss of average ^{18}O enrichment and P^{18}O_4 species during medium P_i -succinate exchange. In these reaction mixtures the concentration of *E. coli* succinyl-CoA synthetase was 44 $\mu\text{g/ml}$ and the concentration of ATP was 3 μM .

distributions resulting from medium or intermediate Pi-HOH oxygen exchange have been calculated on the basis of probability considerations (89). A copy of the Fortran computer program (see Appendix 1) for medium Pi-HOH exchange was kindly supplied by Dr. Hackney and was applied to the medium Pi-succinate exchange catalyzed by succinyl-CoA synthetase. The only conceivable problem in using this program, which was designed to calculate the Pi distribution for medium Pi-HOH exchange and not Pi-succinate exchange, results from the oxygen pool of succinate being much smaller than the pool from water. Accordingly, a succinate molecule that has already been involved in the exchange reaction and contains ^{18}O has a finite probability of reassociation with the enzyme and undergoing exchange a second time. This would result in the enriched Pi species having a distribution different from that predicted by this program. However, Figure 17 shows that the exchange catalyzed by succinyl-CoA synthetase proceeds as a first order process. It therefore appears that the rebinding of exchanged succinate does not become a significant factor in these experiments. It should also be noted that this program is identical to the one used by Bild *et al.* (29) for analysis of medium Pi-succinate exchange. (P.D. Boyer, personal communication).

The distributions of enriched Pi species observed during numerous *E. coli* and pig heart succinyl-CoA synthetase catalyzed exchanges are given in Figures 18 and 19, respectively. These profiles were produced during the

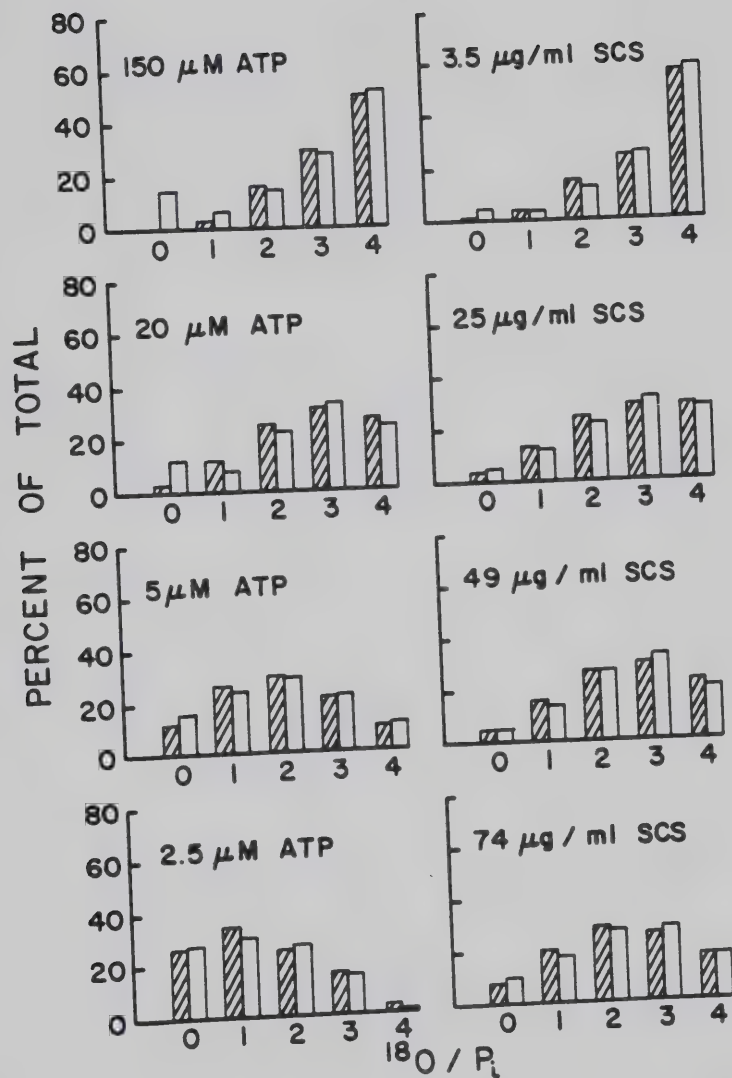


Fig. 18 - Distribution of ^{18}O -labelled P_i species during *E. coli* succinyl-CoA synthetase catalyzed medium P_i -succinate exchange. The reaction mixtures generating the profiles on the left contained 51 $\mu\text{g/ml}$ enzyme and varying concentrations of ATP as shown. The profiles on the right were catalyzed in reaction mixtures containing 5 μM ATP and varying amounts of enzyme as shown. In all cases, the amount of product formed was between 225 to 325 nmoles. \square observed; \square theoretical for P_c of 0.33.

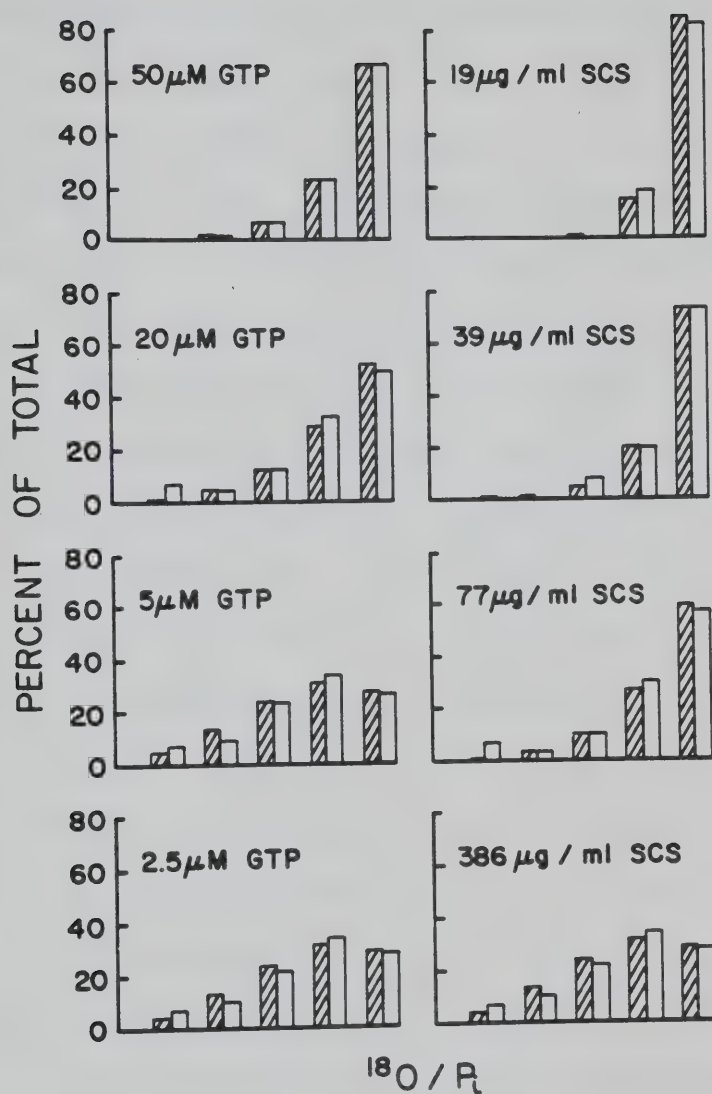


Fig. 19 - Distribution of ^{18}O -labelled P_i species during pig heart succinyl-CoA synthetase catalyzed medium P_i -succinate exchange.. The reaction mixtures producing the profiles on the left contained 386 $\mu\text{g/ml}$ enzyme and varying concentrations of GTP as shown. The profiles on the right were generated in mixtures containing 2.5 μM GTP and varying amounts of enzyme as shown. In all cases, the amount of product formed was between 170 to 270 nmoles. \square observed; ▨ theoretical for P_i of 0.33.

exchange experiments previously described in Figures 13 and 14 for the *E. coli* enzyme, and 15 and 16 for the pig heart enzyme. The distributions on the left side of Figure 18 correspond to the reactions of Figure 13 in which the ATP concentration was varied while the *E. coli* enzyme concentration was held constant at 51 $\mu\text{g/ml}$ (the profiles found when the ATP concentration was varied at the two lower enzyme concentrations are not shown, since neither the relative amount of exchange nor the distribution of P_i species were much different). The right half of Figure 18 shows profiles generated under the conditions described in Figure 14, i.e. 5 μM ATP and varying amounts of *E. coli* succinyl-CoA synthetase. Figure 19 gives the P_i distributions catalyzed during the analogous set of pig heart enzyme experiments, i.e. the exchange reactions of Figures 15 and 16.

Also included in Figures 18 and 19 are the theoretical distributions computed for an exchange process with a P_c of 0.33. Good agreement is observed between the experimental and theoretical profiles for all isotopic P_i species throughout the entire range of exchange conditions. (The observed amount of the P_i species containing no ^{18}O is often greater than predicted but this is likely due to contamination by unlabelled P_i). These results indicate that the exchange catalyzed by succinyl-CoA synthetase can be adequately described by a single process with a unique P_c value and give no evidence for the presence of both dimeric and tetrameric species with altered microscopic rate

constants characterizing significantly different routes of catalysis.

D. Discussion

The experimental results of Figures 13, 14, 15 and 16 demonstrate clearly that the relative amount of oxygen exchange catalyzed by succinyl-CoA synthetase and the nucleotide sensitivity of that exchange are modulated by the concentration of enzyme used in the reaction mixture. Both the *E. coli* and pig heart enzyme catalyze a nucleotide-sensitive or 'cooperative' exchange reaction when assayed at a sufficiently high concentration. The protein concentration range in which the two enzymes produce the cooperative exchange pattern is, however, very different: 50 $\mu\text{g/ml}$ for the *E. coli* enzyme compared to approximately 400 $\mu\text{g/ml}$ for the pig heart species. Bild *et al.* (29) have attributed the 'cooperative' exchange pattern (the increased amount of exchange at low nucleotide concentrations) to catalytic cooperativity, i.e. the interaction of nucleotide at one catalytic site influencing the exchange steps at the other catalytic site. They eliminated enzyme hysteresis or heterogeneity as an explanation for the nucleotide sensitive exchange by means of an analysis of the distribution of ^{18}O -labelled Pi species produced during exchange. When the same analysis was done on the experiments presented in this chapter, it once again indicated that the exchange reaction could be adequately described by a single catalytic route.

However, the striking behavior described here, that is the dependence of a kinetic feature (the cooperativity of the exchange reaction) on the protein concentration used in the experimental study, is often indicative of a system having a protomer-oligomer equilibrium. These kinetic results, in combination with the previously reported concentration dependence of the molecular weight of *E. coli* succinyl-CoA synthetase (48), give substantial support to the concept that succinyl-CoA synthetase may exist in a dimer-tetramer equilibrium. Perhaps the theoretical analysis of the distributions of enriched P_i species is not a sufficiently sensitive probe for the detection of multiple catalytic routes, especially ones catalyzed by a dimer and tetramer in equilibrium.

This approach for assessment of the nature of catalytic events giving rise to substrate modulation of oxygen exchange has previously been applied successfully to oxidative phosphorylation (27) and photophosphorylation (90) with demonstration of a single catalytic pathway. It has also been used to demonstrate the presence of mixed pathways for myosin and actomyosin (91,92,93,94). However, as pointed out by Hackney (89), these were the more favourable cases for the distinction between homogeneous and heterogeneous catalytic routes. He demonstrated that the theoretical distribution of ^{18}O -species produced during medium P_i -HOH (and medium P_i -succinate) exchange has a shallow dependence on P_c in the lower range of P_c values. For example, a P_c

value of 0.3 would be difficult to distinguish from 0.2. Accordingly, a mixed pathway involving two low P_c values would probably be indistinguishable from a homogeneous pathway characterized by one low P_c . Therefore succinyl-CoA synthetase may actually possess a dimer-tetramer equilibrium, but the presence of these two species during exchange would be impossible to detect using the analysis of ^{18}O -Pi distributions if both P_c values were relatively low.

A reasonable model not involving catalytic cooperativity may be offered as an explanation of the oxygen exchange results presented in this chapter. The dimeric and tetrameric forms of succinyl-CoA synthetase are present with significantly different K_m values for ATP but identical or similar P_c values. The dimer has a high K_m and catalyzes little medium Pi-succinate exchange. This form would be responsible for most of the reaction at both high and low ATP concentrations when the enzyme is diluted. However, as the ATP concentration is lowered and/or the protein concentration is raised a larger portion of the reaction is catalyzed by the tetramer which has a low K_m and catalyzes extensive exchange. The P_c value would remain constant even while the flux of the reaction changes from one form to the other. Whether or not the dimer-tetramer equilibrium is pre-existing or is induced by substrates is not known but is unimportant in this discussion. Previous studies have shown that the equilibrium favours the tetramer for the *E. coli* enzyme (48) and lies far towards the dimer for pig heart

succinyl-CoA synthetase (49). It is predictable then, that extensive exchange is catalyzed by the two enzymes at very different protein concentrations. The pig heart enzyme must have a higher concentration before the tetrameric form of the enzyme can catalyze a significant portion of the reaction. Initial rate kinetics which support this model are presented in the next chapter.

V. KINETIC STUDIES ON THE DIMER-TETRAMER EQUILIBRIUM OF SUCCINYL-COA SYNTHETASE

A. Introduction

If the state of aggregation or association of an enzyme affects its kinetic behaviour, then the enzymatic activity *per se* may be used as a probe of protein-protein interactions. In the preceding chapter a model involving self-association was developed for succinyl-CoA synthetase on the basis of oxygen exchange kinetics. It was proposed that both the *E. coli* and pig heart enzymes exist in dimer-tetramer equilibria and that the dimer has a higher K_m for ATP or GTP than the tetramer. If this hypothesis is true, then initial velocity studies should provide further support for the concept. Studies on the effect of protein concentration on the K_m for ATP together with other initial velocity studies that yield information pertinent to the proposed dimer-tetramer equilibrium are presented in this chapter.

B. Materials and Methods

1. Effect of *E. coli* succinyl-CoA synthetase concentration on its K_m for ATP

The enzyme was assayed under the same conditions used in the oxygen exchange reactions of the preceding chapter. The velocity was measured by following the decrease in absorbance at 340 nm accompanying the oxidation of NADH.

This assay is more reliable for initial rate measurements than is the estimation of the Fe^{3+} complex of succinohydroxamate. The two assays, however, consistently showed the same general trends.

2. Effect of KCl on the rate of the E. coli succinyl-CoA synthetase reaction

In these experiments the reaction velocity was assayed by a modification of the direct spectrophotometric method (68) which is based on the increase in absorbance at 232 nm accompanying thioester formation. The exact conditions used were as follows: the mixture contained, at pH 7.2, 60 mM Hepes-NaOH, 10 mM MgCl_2 , 20 mM disodium succinate, 0.20 mM CoA, 150 μM ATP, 5-50 $\mu\text{g/ml}$ *E. coli* succinyl-CoA synthetase and 5-500 mM KCl. Reactions were run at 25°C in a total volume of 1.0 ml.

3. Activity of refolded E. coli succinyl-CoA synthetase in the presence of NEM-modified β

These hybrid enzymes were prepared using the same techniques described in Chapter 3 for the preparation of the NBD-Cl-modified hybrid enzymes, the only difference being that varying amounts of NEM-modified β instead of NBD-Cl-modified α were added to the refolding enzyme. Aliquots of the hybrid enzyme mixtures were assayed using the direct spectrophotometric method (68). NEM was purchased from Sigma Chemical Company. (These hybrid enzyme experiments were carried out by Edward R. Brownie.)

C. Results

1. Effect of *E. coli* succinyl-CoA synthetase concentration on its K_m for ATP

Figure 20 (v versus $[S]$) and Figure 21 ($1/v$ versus $1/[S]$) show the kinetic behaviour of *E. coli* succinyl-CoA synthetase as a function of the concentration of enzyme used in the assay. The kinetic parameters were calculated from Figure 21 (the Lineweaver-Burk plot). It can be seen that, as postulated, the apparent K_m for ATP increases upon enzyme dilution, i.e. the K_m is $38 \mu M$ when the enzyme is assayed at $53 \mu g/ml$ and $200 \mu M$ when the enzyme concentration is $4.4 \mu g/ml$. Furthermore, these experiments revealed an additional unexpected effect of enzyme concentration: V_{max} (expressed as specific activity) increases 6-fold as the enzyme concentration is lowered from 53 to $4.4 \mu g/ml$. These results suggest that the dimer not only has a higher K_m for ATP, but also has a higher turnover number at saturating concentrations of ATP. This same trend (i.e. an increase in specific activity upon enzyme dilution) was observed in the few kinetic experiments that were done on the pig heart enzyme (data not shown). It should also be noted that the double-reciprocal plot for the *E. coli* enzyme is linear when the enzyme concentration is high but exhibits positive cooperativity when the concentration of enzyme is reduced.

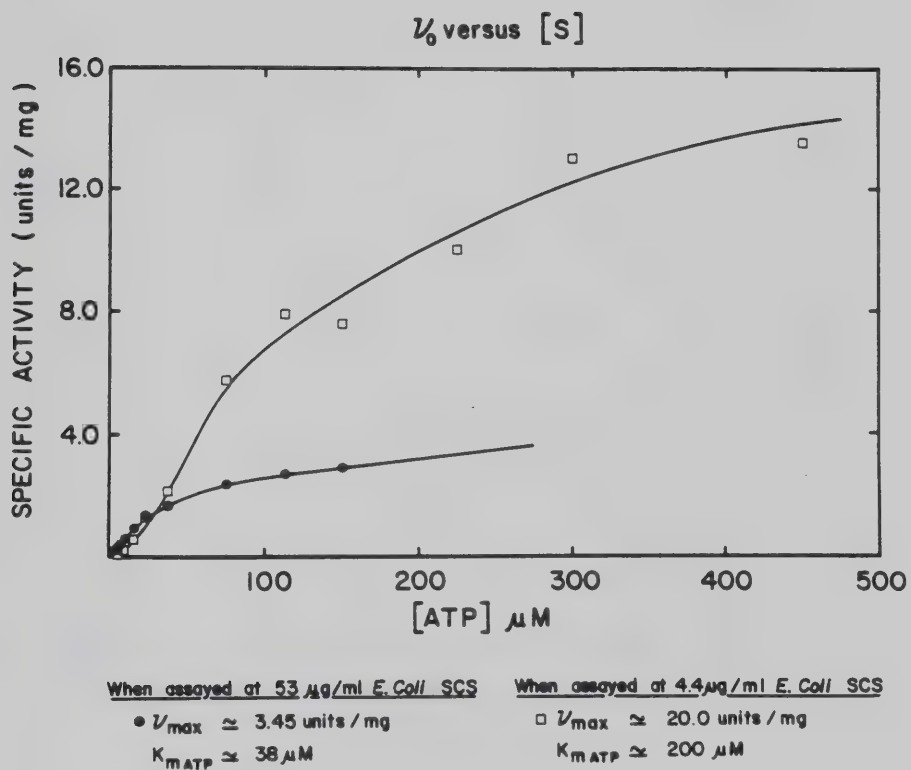


Fig. 20 - Effect of *E. coli* succinyl-CoA synthetase concentration on its kinetic parameters: the Michaelis-Menten plot Reaction conditions are as described in the text. SCS=succinyl-CoA synthetase.

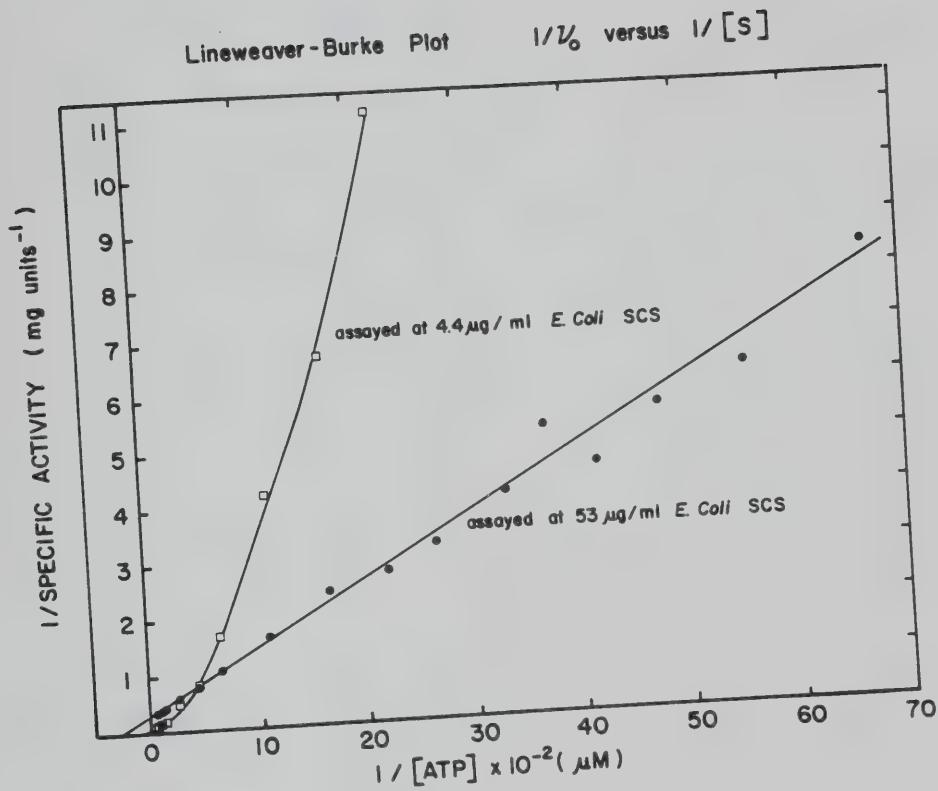


Fig. 21 - Effect of E. coli succinyl-CoA synthetase concentration on its kinetic parameters: the Lineweaver-Burke plot. Reaction conditions are as described in the text. SCS=succinyl-CoA synthetase.

2. Effect of KCl on the rate of the *E. coli* succinyl-CoA synthetase reaction

There is no previous reference in the literature to an enzyme concentration effect on the kinetics of *E. coli* succinyl-CoA synthetase. This oversight may be traced to the fact that most kinetic investigations of this enzyme have made use of the direct spectrophotometric assay. The medium used for this assay contains a much lower concentration of KCl than that for the coupled assay based upon NADH oxidation which was used in both the oxygen exchange experiments and the kinetic experiments described above. The high salt concentration in the coupled assay is present because of the need to neutralize the high concentration (0.48 M) of hydroxylamine hydrochloride with KOH. Accordingly, the concentration of KCl in the NADH assay is approximately 0.5 M higher than in the direct assay. It is well known that neutral salts may have profound effects on the conformational stability of proteins, in some cases inducing dissociation (95, 96). It is possible, then, that the *E. coli* enzyme remains for the most part associated as a tetramer in the direct assay whereas in the NADH assay the extra salt may shift the equilibrium towards the dimer, allowing its influence to be felt on the kinetics. If succinyl-CoA synthetase is dissociated to dimers by salt, and if the dimers do have a higher specific activity at saturating concentrations of ATP, then the addition of KCl to the direct assay should result in an increase in activity. Also,

the extent of activation should depend on the enzyme concentration used in the assay.

The results shown in Figure 22 are consistent with these proposals. It can be seen that KCl first activates and then inhibits *E. coli* succinyl-CoA synthetase, and that the activation is greater (123% of the original activity at 50 mM KCl) when the enzyme concentration is 5.0 $\mu\text{g/ml}$ than when the concentration is increased to 50.0 $\mu\text{g/ml}$ (106% of the original activity at 50 mM KCl).²

3. Activity of refolded *E. coli* succinyl-CoA synthetase in the presence of NEM-modified β

The results of a hybrid enzyme experiment done several years ago in our laboratory remained enigmatic until the time when the kinetic experiments led to the dimer-tetramer hypothesis. These data are presented in Figure 23. The addition of increasing amounts of NEM-modified β to the refolding enzyme results in an initial activation (which peaks at a modified/native β subunit ratio of approximately 2.3) followed by inactivation of the enzyme. As discussed

²This activation, however, is much less than that observed in the coupled assay based on NADH oxidation, i.e. Figure 20 shows a 6-fold increase in V_{max} as the enzyme concentration is lowered from 53 to 4.4 $\mu\text{g/ml}$. Since this discrepancy may have been due to the added presence of hydroxylamine in the coupled assay, further experiments have been carried out to investigate this possibility using an assay which measures the release of ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Wolodko, W.T. - unpublished). The results of these studies indicate that the presence of hydroxylamine cannot account for the large activations seen in Figure 20. The only remaining difference between the two assay conditions is the presence of P_i and its effect on the kinetics of succinyl-CoA synthetase is currently under investigation.

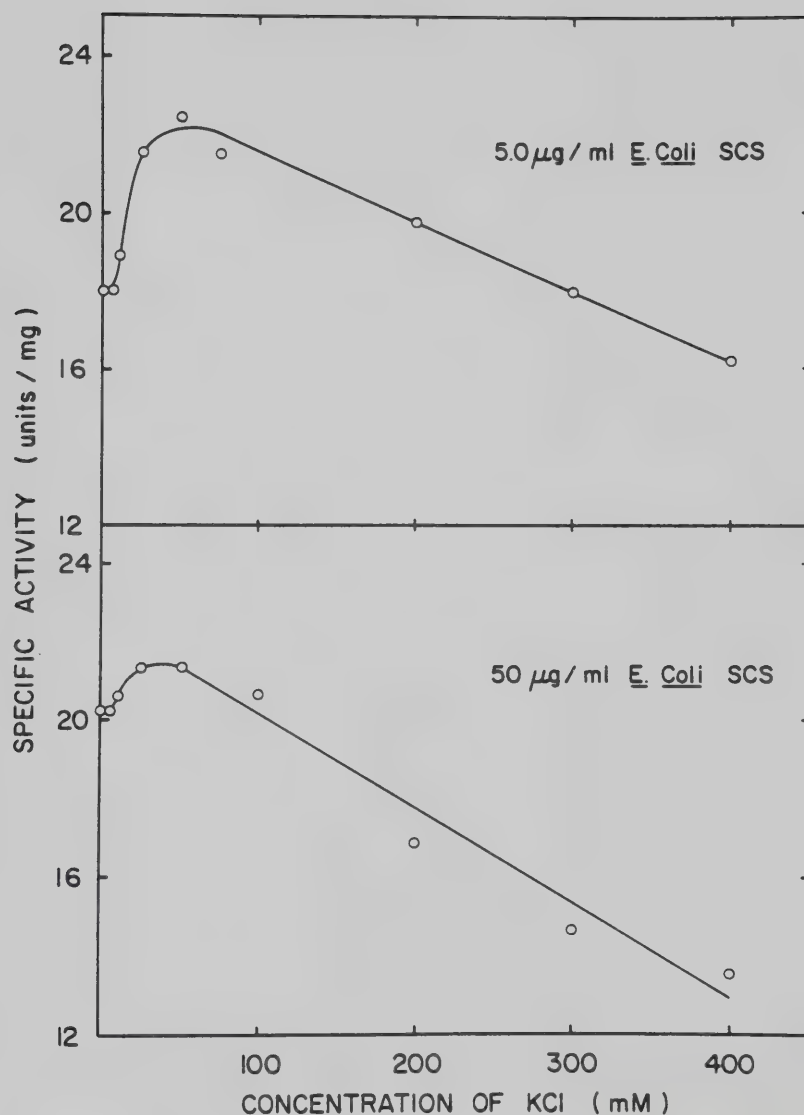


Fig. 22 - The influence of KCl on the activity of *E. coli* succinyl-CoA synthetase at two different enzyme concentrations. The enzyme activity was measured using the direct assay as described in the text. SCS=succinyl-CoA synthetase.

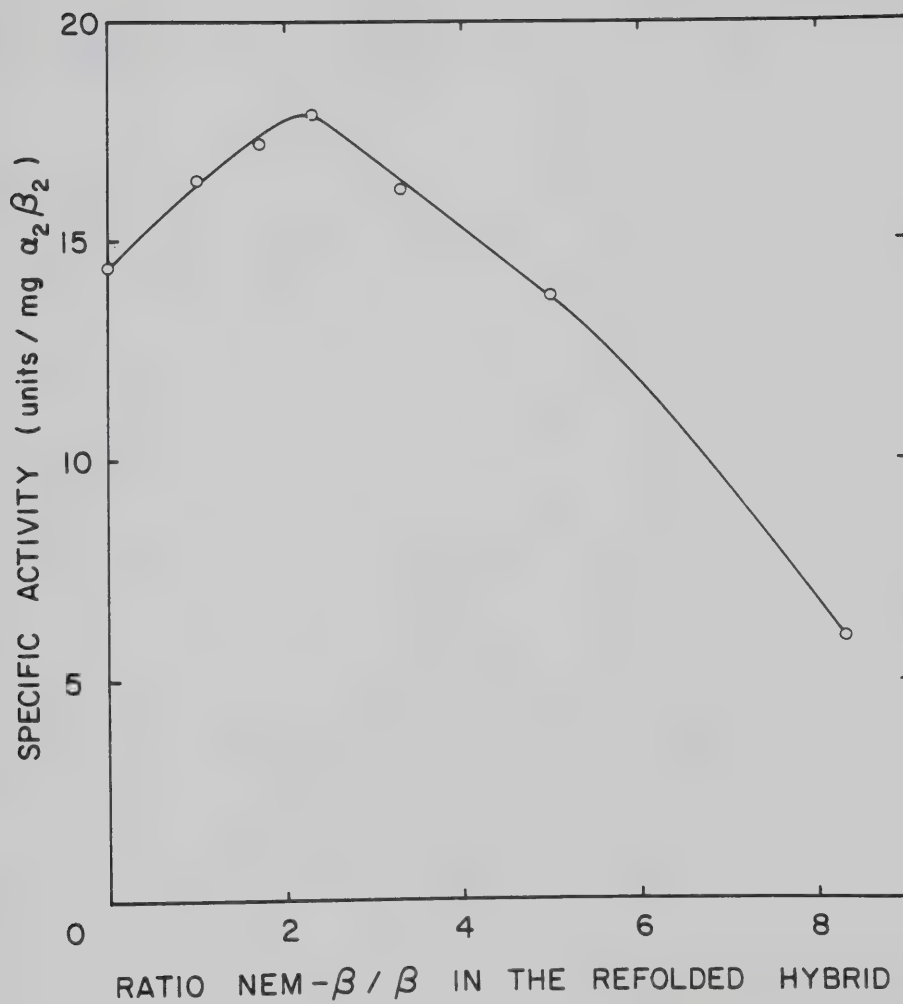


Fig. 23 - The activity of refolded E. coli succinyl-CoA synthetase in the presence of increasing amounts of NEM modified β . The refolding and assay conditions are as described under Materials and Methods.

below, this phenomenon can be readily explained through a consideration of the proposed dimer-tetramer equilibrium for *E. coli* succinyl-CoA synthetase.

D. Discussion

In the preceding chapter a model was developed to explain the oxygen exchange kinetics catalyzed by succinyl-CoA synthetase. It was proposed that both the pig heart and *E. coli* enzyme are in dimer-tetramer equilibria in the assay and that the dimer has a higher K_m for ATP. The initial velocity studies shown in Figures 20 and 21 of this chapter support this hypothesis since the K_m for ATP increases as the *E. coli* enzyme concentration is lowered (i.e. as the equilibrium is shifted more towards the dimer).

The observed increase in V_{max} at the lower enzyme concentration was unexpected. It has been shown that the tetrameric *E. coli* enzyme exhibits half-of-the-sites reactivity (or at least strong negative cooperativity) with respect to phosphorylation (44,18). If the tetramer maintains this half-site reactivity during catalysis then the number of available active sites would be expected to double upon dissociation of the enzyme into two independent dimers (each containing an active site). It follows that V_{max} (in terms of units/mg enzyme) would double, assuming that the catalytic activities of active sites in the dimer and tetramer are equivalent. According to Bild's theory on catalytic cooperativity (29), the interaction of ATP with

one catalytic site in the tetramer may increase flux through rate-limiting steps at the other site, resulting in improved catalytic efficiency. If this theory were correct, then V_{max} might increase upon enzyme dissociation but it would not be expected to reach twice the value found for the associated enzyme. In other words, the sum of the activity catalyzed by the two independent sites in the dimers would be less than twice the activity catalyzed by the one more efficient site in the tetramer. However, it was found (Figures 20 and 21) that as the enzyme is diluted from 53 $\mu\text{g/ml}$ to 4.4 $\mu\text{g/ml}$ the value for V_{max} increases 6-fold. (It might have increased even further if a lower enzyme concentration had been tested.) Therefore, the active site in the dimer seems to be more efficient than the one in the tetramer, at least under the conditions of this assay. Although these results do not exclude the possibility of communication between active sites in the tetramer, they indicate that these subunit interactions do not result in a catalytic site that is more efficient than the independent site in the dimer. In fact, an obvious interpretation is that the active site in the tetramer is in a constrained or inhibited conformation relative to the site free from subunit interactions. Upon reconsideration of the model proposed in the last chapter, it is not surprising that the active site is more efficient in the dimer. It was postulated that the tetrameric form of succinyl-CoA synthetase not only has a lower K_m for ATP but also catalyzes the reaction with extensive oxygen exchange

as compared to the dimer. If the exchange step that is affected by the association state is rate-limiting, then improved catalytic efficiency would result.

The oxygen exchange kinetics catalyzed by succinyl-CoA synthetase remain compatible with the dimer-tetramer model, even though V_{max} , as well as K_m , change with the association state of the enzyme. The important point is that the tetramer (which is postulated to catalyze extensive exchange) must be responsible for a significant portion of the reaction at low ATP concentrations. Figure 20 shows that, even though V_{max} is much higher when the enzyme is diluted, at low ATP concentrations more of the reaction would be catalyzed by the form predominant at high enzyme concentration (the tetramer) than by the form produced upon dilution (the dimer).

The cooperativity of the double reciprocal plot (Figure 21) at the lower *E. coli* enzyme concentration is most likely due to the presence of both dimer and tetramer in equilibrium. When the higher enzyme concentration is used, the proportion of dimer may be so low as to be undetectable in the kinetics, giving rise to the linear plot. In order to produce cooperative kinetics, the dimer-tetramer equilibrium must either be slower than the maximum velocity of the reaction (hysteretic cooperativity) or if the equilibrium is rapid it must be shifted by the presence of ATP (this is analogous to the shift in equilibrium envisioned in the concerted model of cooperativity (12)). It is not possible

to distinguish between these two possibilities at this point.

The initial velocity studies done on the pig heart enzyme were not as detailed as those on the *E. coli* enzyme. However, they were sufficient to establish that V_{max} increased upon enzyme dilution (insufficient data were obtained for accurate estimation of K_m). This result is encouraging in that it suggests that the pig heart enzyme also is subject to a dimer-tetramer equilibrium (as postulated in the preceding chapter) with the dimer being more active.

Figure 22 shows that 50 mM KCl activates the *E. coli* enzyme in the direct assay. This result, together with the knowledge that the dimer is more active, raises the possibility that KCl activation may be attributable to promotion of dissociation of the enzyme. The fact that the magnitude of activation by KCl is less at higher enzyme concentrations supports this conclusion (i.e., higher enzyme concentrations and salt would shift the equilibrium in opposite directions). The activating effect of 50 mM KCl on succinyl-CoA synthetase from soybean and tobacco has been reported previously (97,98) but no explanation was offered for the effect. It is possible that dissociation is also responsible for these activations.

The increase in specific activity (units/mg $\alpha_2\beta_2$) observed in the hybrid enzyme experiment of Figure 23 may also be due to a dissociating effect. The modified subunit

which was added in increasing amounts to the refolding *E. coli* enzyme had been exposed to the sulfhydryl reagent, NEM. There have been several reports in the literature that sulfhydryl modifications by mercurials can induce dissociation of the *E. coli* enzyme (46,77,99).³ It is an attractive possibility then that the activation is attributable to the modified subunit refolding into the tetramer with the equilibrium displaced towards the dimer, i.e. $\alpha_2\beta\beta' \longrightarrow \alpha\beta + \alpha\beta'$. The presence of significant amounts of dimer would lead to the initial activation. Decreased specific activity at higher proportions of β' is of course expected because of the increasing proportions of modified, inactive dimer.

The initial velocity studies presented in this chapter are consistent with the dimer-tetramer model as an explanation for the oxygen exchange kinetics of succinyl-CoA synthetase. However, the interpretation of these kinetic measurements would be strengthened by independent studies of protein-protein interactions using physical techniques. These experiments were carried out with the aim of evaluating the dimer-tetramer equilibrium under different conditions and are presented in the next chapter.

³Treatment with NEM, however, does not cause sufficient dissociation to result in a significant change in the sedimentation coefficient of the modified enzyme (Bridger, W.A. - unpublished observation.)

VI. PHYSICAL STUDIES ON THE DIMER-TETRAMER EQUILIBRIUM OF SUCCINYL-CoA SYNTHETASE

A. Introduction

A dimer-tetramer equilibrium for succinyl-CoA synthetase has been proposed in the preceding chapters solely on the basis of enzyme kinetics (oxygen exchange and initial velocity studies). Physical studies are presented in this chapter which complement the kinetic experiments. The equilibrium was examined using both mass migration methods (gel chromatography and sedimentation velocity) and an equilibrium method (sedimentation equilibrium). These studies concentrated mainly on the *E. coli* enzyme. The molecular weight of the enzyme was determined by means of gel filtration under conditions where active catalysis was occurring. The effects of individual components of the reaction mixture on the equilibrium were studied using all three techniques.

B. Materials and Methods

1. Gel filtration

A column of Sephacryl S-200 superfine (1.5 cm x 90 cm) was calibrated with the following molecular weight standards: ribonuclease A (M.W. 13,700), chymotrypsinogen A (M.W. 25,000), ovalbumin (M.W. 45,000) and pig heart succinyl-CoA synthetase (M.W. 77,000). The void volume was determined using ATP-citrate lyase (M.W.=440,000). The

equilibrating and eluting buffer was 60 mM HEPES, 10 mM MgCl₂ at pH 7.2. The column was run at 25°C with a flow rate of 0.4 ml/min and 1 ml fractions were collected. The molecular weight of *E. coli* succinyl-CoA synthetase was examined with the following additions made to the buffer:

Buffer A - no additions

Buffer B - 150 μ M ATP

Buffer C - 400 μ M ATP, 10 mM succinate, 100 μ M CoA and 100 μ M DTT (i.e. all substrates at concentrations above their respective K_m values).

Buffer D - 150 μ M ATP, 20 mM succinate, 200 μ M CoA, 5 mM Pi, 1.0 mM phosphoenolpyruvate, 480 mM hydroxylamine hydrochloride, 400 μ M NADH, pyruvate kinase (40 units/ml), lactate dehydrogenase (110 units/ml) (i.e. the reaction mixture in which oxygen exchange was measured).

Buffer E - the same as D but 2.5 μ M ATP instead of 150 μ M ATP.

The *E. coli* enzyme was applied to the column at a concentration of 100 μ g/ml (total volume=1 ml) except for Buffer D and E where it was also loaded at 10 μ g/ml. The enzyme was detected as it emerged from the column by taking an aliquot of each fraction and assaying for activity in the direct assay (68).

Sephacryl S-200, ribonuclease A, chymotrypsinogen A and ovalbumin were purchased from Pharmacia Fine Chemicals. Rat liver ATP-Citrate lyase was purified by Mary Packer according to the procedure of Linn and Srere (100).

2. Sedimentation Velocity

The sedimentation coefficient of *E. coli* succinyl-CoA synthetase was determined at three enzyme concentrations (0.2, 0.4 or 0.6 mg/ml), all three concentrations being examined in the presence of either low salt, high salt or low salt plus Pi. The buffer used was 60 mM HEPES, 10 mM MgCl₂, 0.1 mM EDTA, pH 7.2 containing either 0.05 M KCl, 0.50 M KCl or 0.05 M KCl plus 5 mM Pi. The sedimentation velocity determinations were carried out at 20°C and 60,000 rpm in an analytical ultracentrifuge equipped with a photoelectric scanner. The enzyme samples at the appropriate concentration were loaded (using plastic tipped pipettes) into double sector cells along with the corresponding buffer. The cells were scanned (235 nm) at 8 minute intervals for approximately 80 minutes. Sedimentation coefficients were determined from the rate of movement of the inflection point of the boundary. Representative tracings are shown in Figure 24. The slopes of the log y versus time plots were determined by linear regression analysis using the Texas Instruments SR-51 calculator. $S_{obs.}$ and $S_{20,W}$ were calculated according to the equations described in reference (101). A partial specific volume of 0.74 cm³/g was used for the *E. coli* enzyme (48).

The enzyme was prepared for the sedimentation velocity experiments by first phosphorylating it in the presence of 400 μ M ATP (incubated for 15 minutes at 25°C) followed by exhaustive dialysis against buffer (60 mM HEPES, pH 7.2)

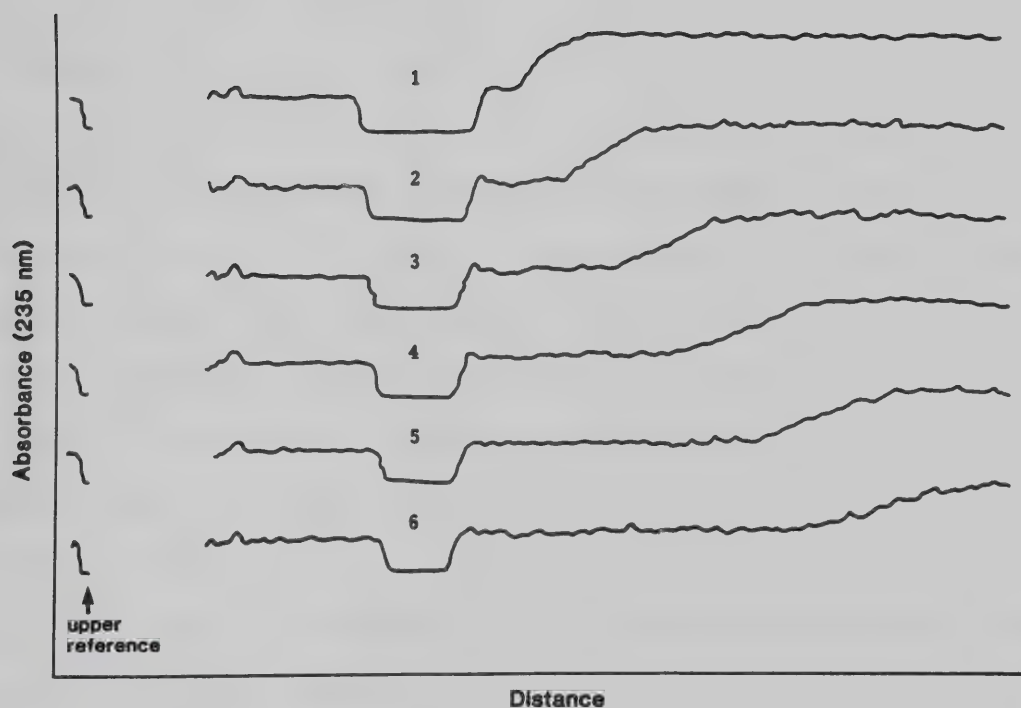


Fig. 24 - Densitometer tracings of absorption patterns observed during the sedimentation of *E. coli* succinyl-CoA synthetase. The tracings represent the optical density at 235 nm in the cell as a function of distance. The enzyme was loaded at a concentration of 0.2 mg/ml and was sedimented in a buffer containing 60 mM HEPES, 10 mM MgCl_2 , 0.1 mM EDTA, 0.5 M KCl. The cells were scanned at 8 minute intervals.

containing Dowex Chelex 100 resin to remove heavy metal contaminants. The enzyme was then diluted into and dialyzed against the appropriate buffer solution (i.e. low salt, high salt, or low salt plus Pi).

3. Sedimentation Equilibrium

The molecular weight determinations were performed by conventional low-speed sedimentation equilibrium methods. The runs were monitored at 280 nm using the photoelectric scanner. The enzyme was loaded into double sector cells (using plastic tipped pipettes) at concentrations between 0.2 and 0.3 mg/ml; the experiments were performed at rotor speeds between 9,000 and 15,000 rpm and at a temperature of 20°C. The data were analyzed using an APL program written for the Amdahl Computer which calculates apparent weight-average molecular weight as a function of concentration from the observed dependence of $\ln(\text{concentration})$ on r^2 . The partial specific volume of 0.74 cm³/g was used (48).

The enzyme was prepared for these sedimentation equilibrium experiments in the same way as for the sedimentation velocity experiments. The same buffers were used, i.e. 60 mM HEPES, 10 mM MgCl₂, 0.1 mM EDTA, pH 7.2 plus one of the following additions: 0.05 M KCl, 0.50 M KCl or 0.05 M KCl plus 5 mM Pi.

C. Results

1. Gel Filtration

Bild *et al.* (29) examined the molecular weight of the pig heart enzyme by gel chromatography under assay conditions and found no evidence for the tetrameric form of the enzyme. The same result was found under the buffer conditions and loading concentrations used in these experiments, i.e. the pig heart enzyme ran as a dimer of molecular weight 77,000. Therefore, the pig heart enzyme was used as one of the standards and efforts were concentrated on detecting the presence of the dimeric form of *E. coli* succinyl-CoA synthetase. Figure 25 shows the elution profile of the *E. coli* enzyme under different conditions. The top four arrows indicate the positions where the standards elute and the lower two arrows indicate positions of the *E. coli* dimer (M.W.=68,000) and tetramer (M.W.=136,000) as predicted from the standard curve. It can be seen that the *E. coli* enzyme activity comes off the column at the position expected for the tetramer and that the profile is not changed significantly by the addition of either 150 μ M ATP alone (Buffer B) or the combination of 400 μ M ATP, 10 mM succinate, 100 μ M CoA, 100 μ M DTT (Buffer C). Since the enzyme proceeds down the column at a faster rate than its product, the last set of additions results in the enzyme being eluted under the initial velocity reaction conditions found in the direct assay. The elution profile also remained relatively unchanged when the enzyme was run through the

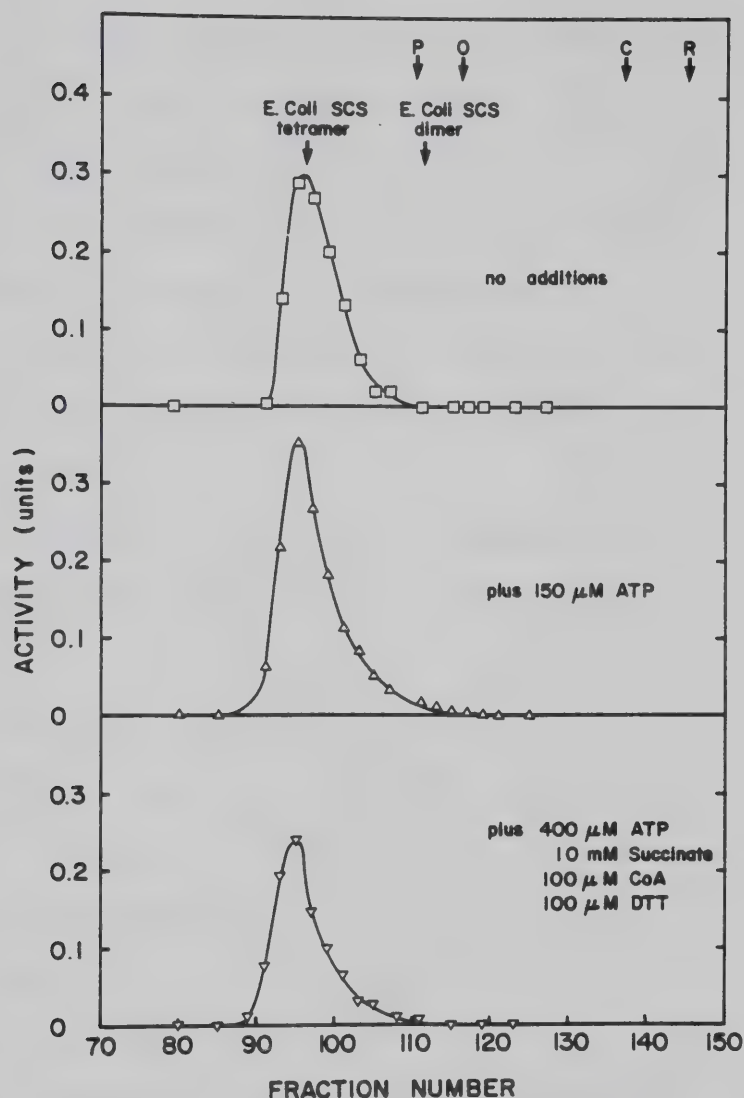


Fig. 25 - Sephacryl S-200 gel filtration of *E. coli* succinyl-CoA synthetase. P=pig heart succinyl-CoA synthetase, O=ovalbumin, C=chymotrypsinogen, R=ribonuclease. The *E. coli* succinyl-CoA synthetase was loaded at a concentration of 100 μ g/ml (total volume=1 ml) and was eluted with 60 mM HEPES, 10 mM $MgCl_2$, pH 7.2 containing the additions shown. (i.e. Buffers A, B and C as described under methods)

column under the conditions found in the exchange reaction mixture, i.e. when it was eluted with Buffer D (Figure 26). When the enzyme was loaded at one-tenth the concentration under the same conditions the peak broadened only slightly (also shown in Figure 26). Changing the concentration of ATP in the elution buffer from 150 μ M to 2.5 μ M (Buffer E) had no effect on the profile at either enzyme loading concentrations.

The skewing of the *E. coli* enzyme activity peak towards the dimeric position under all the conditions examined may indicate the presence of a small amount of dimer in equilibrium with the tetramer (especially since the standard eluted with symmetrical peaks). Within the limits of detection, however, the equilibrium does not appear to be shifted significantly by any of the additions made to the elution buffer.

2. Sedimentation Velocity

The effects of individual components of the oxygen exchange reaction mixture on the dimer-tetramer equilibrium of *E. coli* succinyl-CoA synthetase were examined by sedimentation velocity studies. A low concentration of salt (0.05 M KCl) was included in all the buffers to reduce non-ideal interactions (primary charge effects) and EDTA was added to remove heavy metals. The effect of raising the KCl concentration to 0.50 M was examined since this amount of salt is carried into the exchange reaction mixture along with the hydroxylamine. The product P_i , at a concentration

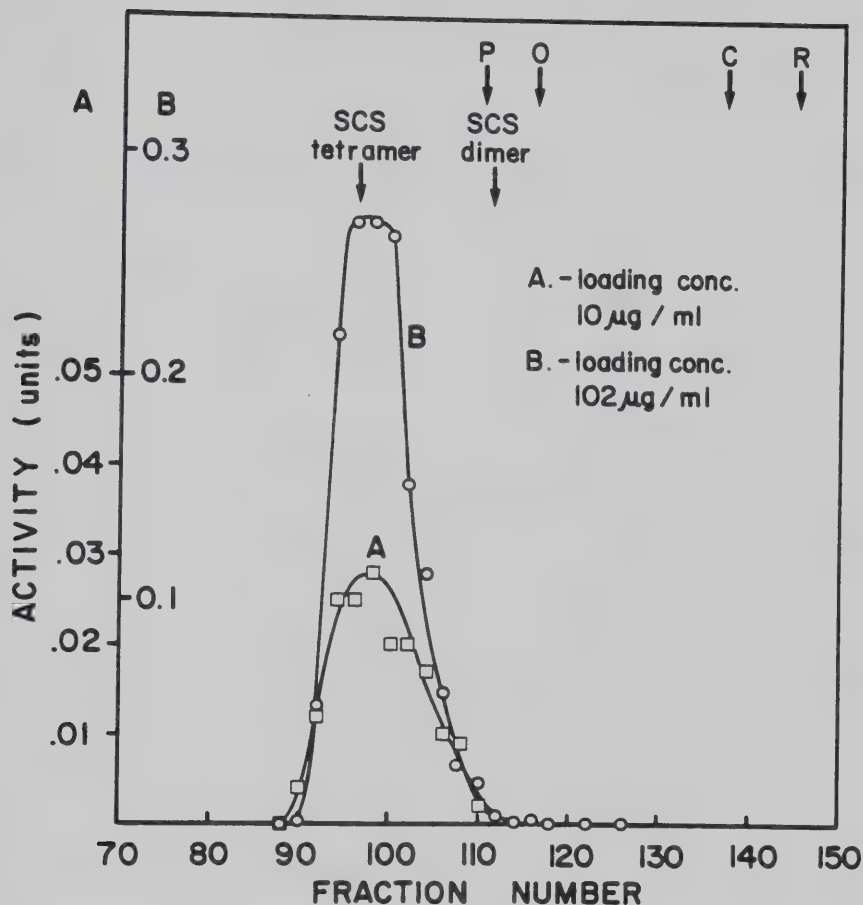


Fig. 26 - Sephacryl S-200 gel filtration of *E. coli* succinyl-CoA synthetase. P=pig heart succinyl-CoA synthetase, O=ovalbumin, C=chymotrypsin, R=ribonuclease. The enzyme was loaded in a total of 1 ml at the concentrations shown and was eluted with Buffer D (as described under methods).

of 5 mM, is present during the exchange and its influence on the sedimentation coefficient was tested. However, the effects of the substrates ATP and CoA could not be examined because of their high absorbance at 235 nm. Also, succinate was not tested since it would complicate the results by promoting dephosphorylation of the enzyme.

The results presented in Table 1 show that the sedimentation coefficient ($S_{20,W}$) of the *E. coli* enzyme remains relatively unchanged in the presence of 0.05 M KCl or 0.05 M KCl plus 5 mM Pi, whether the enzyme is loaded at 0.2, 0.4 or 0.6 mg/ml. However, with the addition of 0.50 M KCl the sedimentation coefficient decreases with decreasing enzyme concentration. This finding suggests that the higher salt and lower enzyme concentrations act in concert to shift the equilibrium more towards the dimer.

3. Sedimentation Equilibrium

The gel filtration experiments demonstrated that although *E. coli* succinyl-CoA synthetase may have a dimer-tetramer equilibrium, the position of the equilibrium is far towards the tetramer, even under conditions of active catalysis. Sedimentation equilibrium may be expected to provide a valuable technique for the study of this equilibrium since, when the photoelectric scanner is used, it gives the concentration dependence of molecular weight in a very low enzyme concentration range, i.e. where a significant proportion of the enzyme may exist as a dimer. The fact that previous sedimentation equilibrium studies on *E. coli*

Table 1

The sedimentation coefficient of *E. coli* succinyl-CoA synthetase as a function of enzyme concentration and buffer additions. The enzyme was sedimented in a buffer containing 60 mM HEPES, 10 mM MgCl₂, 0.1 mM EDTA plus the additions shown above. See text for further experimental details.

 $S_{20,w}$

Buffer [E] Additions	0.2 mg/ml	0.4 mg/ml	0.6 mg/ml
0.05 M KCl	6.48	6.52	6.40
0.50 M KCl	5.99	6.57	6.75
0.05 M KCl + 5 mM Pi	6.38	6.46	6.40

succinyl-CoA synthetase have shown a marked dependence of molecular weight on enzyme concentration (48) indicates that this may be the technique best suited for the study of the *E. coli* enzyme equilibrium.

The sedimentation equilibrium experiments which I carried out (representative data are given in Figure 27) show that, when 0.05 M KCl is the only addition to the buffer, the weight-average molecular weight reaches the value expected for the tetrameric molecular weight (140,000) at high enzyme concentrations but at low concentrations (<0.5 mg/ml) the enzyme appears to dissociate. (Krebs *et al.* (48) had used similar conditions and obtained the same result.) The effect of raising the salt concentration to 0.50 M was investigated and it can be seen (Figure 27) that the apparent weight-average molecular weight decreases over the entire enzyme concentration range. This down-shift in molecular weight supports the hypothesis that salt promotes dissociation of the enzyme. It is also evident from Figure 27 that inorganic phosphate acts to stabilize the tetrameric state of the enzyme, i.e. when 5 mM Pi and 0.05 M KCl are added to the buffer the weight-average molecular weight remains relatively constant at 140,000 over all but the lowest portion of the enzyme concentration range. The influences that CoA, ATP and succinate might have on the equilibrium could not be investigated because of the complicating factors mentioned above in the section on sedimentation velocity.

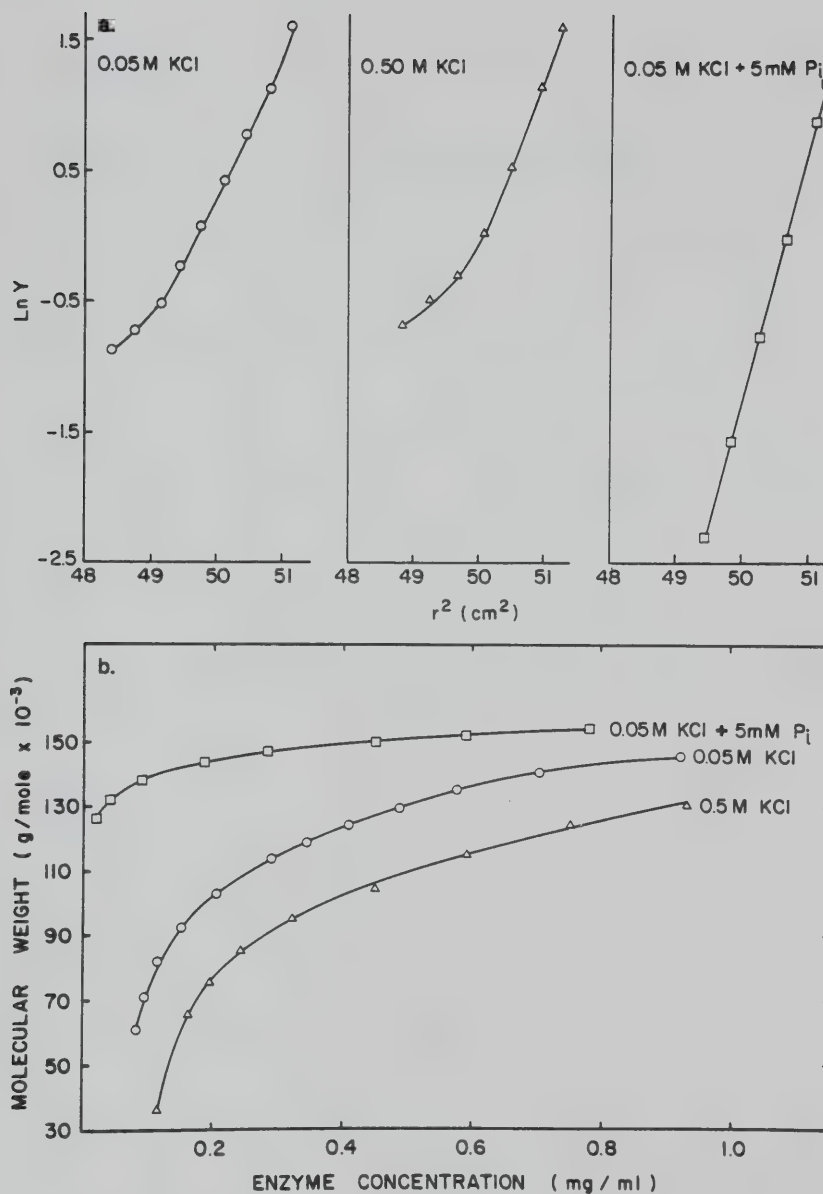


Fig. 27 - Sedimentation equilibrium of *E. coli* succinyl-CoA synthetase. (a) Representative plot of $\ln y$ versus r^2 derived from a run carried out at an enzyme concentration of 0.2 mg/ml and a rotor speed of 15,000 rpm. (b) Concentration dependence of weight-average molecular weight of succinyl-CoA synthetase from the data in part (a). The buffer consisted of 60 mM HEPES, 10 mM $MgCl_2$, 0.1 mM EDTA plus the additions shown. Similar plots were obtained when other enzyme loading concentrations and rotor speeds were used.

D. Discussion

A knowledge of the state of association of an enzyme under assay conditions is essential for an understanding of the molecular basis of the kinetic properties. Several techniques including sedimentation equilibrium, sedimentation velocity and gel filtration were used to measure the molecular weight of *E. coli* succinyl-CoA synthetase under different conditions (including initial velocity reaction conditions) and protein concentrations. Before discussing the results of these experiments, I wish to mention that I initially attempted to measure the oligomeric state of both the *E. coli* and pig heart enzyme using the technique termed 'active enzyme centrifugation' (102). This method involved the layering of a narrow band of enzyme solution on top of a centrifuge cell filled with the coupled assay mixture. The absorbance of the cell was monitored at 340 nm and the position of the active enzyme was marked by a decrease in absorbance due to the loss of NADH. The sedimentation velocities of the *E. coli* and pig heart enzymes were found to be identical using this technique. These results are contradictory to those obtained from the 'active enzyme gel chromatography' method presented in this chapter, which shows that the *E. coli* and pig heart enzyme run mainly as a tetramer and dimer, respectively. There may be two sources of error in the sedimentation experiment. First, it is known that associating-dissociating protein systems may be sensitive to the pressures exerted upon them in

centrifugation experiments (103). Second, since the coupling enzymes used in the assay are both of higher molecular weight than the succinyl-CoA synthetase tetramer, they would sediment ahead of the enzyme of interest. It is likely that the latter consideration is the true source of the apparent equivalence of behaviour of the two succinyl-CoA synthetase preparations in the 'active enzyme centrifugation' experiment since the pig heart and *E. coli* enzyme appeared to travel with exactly the same sedimentation coefficient (Sobs.), and this value for Sobs. is higher than that determined for the *E. coli* enzyme in the normal sedimentation velocity experiment. (It is difficult to calculate $S_{20,W}$ for comparison from the active enzyme experiment because of the many components in the reaction mixture.)

The results of gel filtration experiments revealed that the *E. coli* enzyme largely retains its tetrameric state, even during active catalysis. The assymetric shape of the activity peaks (Figure 25), together with the broadening of the peak upon enzyme dilution (Figure 26), may indicate the presence of a small amount of dimer in equilibrium with the tetramer. However, this equilibrium is barely detectable by this zonal analysis. (Zonal chromatography entails the migration of a small zone of solute (enzyme)). The frontal method of analysis, which involves the migration of an initially sharp boundary between solvent and solution with a plateau of original composition being preserved, might have been more informative since a mixture of enzyme oligomeric

states can be detected by resolution of the initially sharp boundary into several boundaries. The composition can be estimated from the relative sizes of these boundaries. However, this type of analysis would have involved the use of excessive amounts of succinyl-CoA synthetase and elution buffer. Therefore, the techniques of sedimentation velocity and equilibrium were examined because of their potential sensitivity and the small sample size required.

The sedimentation velocity experiments presented in Table 1 show that *E. coli* succinyl-CoA synthetase travels with a relatively constant sedimentation coefficient ($S_{20,W} = 6.4$ to 6.5) in buffer containing either 0.05 M KCl or 0.05 M KCl plus 5 mM Pi, with loading concentrations ranging from 0.2 to 0.6 mg/ml. This suggests that the enzyme remains stable in its tetrameric state under these conditions. (In order to calculate the molecular weight corresponding to this sedimentation coefficient, the diffusion coefficient of the enzyme would have to be determined.) However, when the salt concentration is raised to 0.50 M KCl, the sedimentation coefficient becomes dependent on the loading concentration of the enzyme. It varies from 5.99 at a loading concentration of 0.2 mg/ml to 6.75 at 0.6 mg/ml. This concentration dependence of $S_{20,W}$ suggests that high salt and low enzyme concentrations act together to promote dissociation of the enzyme to dimers. (The increased value for $S_{20,W}$ at the loading concentration of 0.6 mg/ml may be due to KCl also causing a conformational change in the

intact tetramer.) These sedimentation velocity results are compatible with the initial velocity kinetic experiments, i.e. it was postulated that the activating effect of KCl is due to a dissociation of the enzyme into dimers.

The sedimentation equilibrium method was the most informative of all the physical techniques investigated. It revealed (Figure 27) a definite dependence of the apparent molecular weight upon the enzyme concentration of *E. coli* succinyl-CoA synthetase. In agreement with the mass migration methods, it shows that the enzyme exists predominantly as a tetramer of overall molecular weight near 140,000 when at high concentrations (>1 mg/ml). However, it can be seen that the lines do not extrapolate to the dimeric molecular weight of 70,000. As suggested by Krebs & Bridger (48), this could be due to a further dissociation of the enzyme into subunits caused by heavy metal contaminants. An increase in KCl concentration produced the expected decrease in molecular weight over the entire concentration range, i.e. it shifts the equilibrium towards the dimer. An unexpected result was the stabilization of the tetramer by 5 mM Pi. Inorganic phosphate is known to induce conformational changes in many proteins (104), and in the case of glutamate dehydrogenase it affects the association-dissociation equilibrium (105). In retrospect then, this stabilization is not so surprising, especially considering that both the *E. coli* and pig heart enzyme retain their activity longer when stored in phosphate buffers.

Sedimentation equilibrium studies were also done on the pig heart enzyme in an attempt to detect the presence of some tetramer. However, the results were complicated by the fact that the pig heart enzyme is much less stable than the *E. coli* enzyme and the majority of the activity is lost over the duration of the experiment.

It is a pity that the effect of ATP on the dimer-tetramer equilibrium could not be examined using the sensitive sedimentation equilibrium technique since an ATP-induced shift in the equilibrium would explain the cooperative kinetic patterns seen in the preceding chapter. The gel filtration method gave no indication (within the substantial limits of detection) of an effect of ATP on the equilibrium (Figure 25). However, fluorescence polarization studies on a 'lightly' dansylated derivative of *E. coli* succinyl-CoA synthetase have revealed that 5 mM ATP (when added to the enzyme in HEPES buffer, pH 7.2 at 0.5 M KCl) causes a dramatic drop in polarization (J.S. Nishimura, personal communication). This may indicate that ATP does actually shift the dimer-tetramer equilibrium. It is also interesting to note that Nishimura did not observe this decrease in polarization with ATP when the enzyme was in phosphate buffer. This may be due to the stabilization of the tetramer by phosphate, as was suggested by the sedimentation equilibrium experiments. However, if the gel filtration results can be trusted and ATP does not affect the oligomeric state of the enzyme, then the cooperative

kinetics must be due to hysteresis, i.e. the dimer-tetramer equilibrium must be slower than the maximum velocity of the reaction. As discussed by Frieden and Nichol (106), it is not unreasonable to expect that the association-dissociation reaction of an enzyme may be rate limiting in the kinetic behaviour.

In summary, physical techniques have given supporting evidence for the dimer-tetramer equilibrium for *E. coli* succinyl-CoA synthetase and have revealed that, under all conditions tested, the equilibrium is far towards the tetramer. The equilibrium, if it does actually exist for the pig heart enzyme, must lie far on the side of the dimer since the tetrameric state was not detected by any of the physical techniques.

VII. COMPARATIVE STUDIES ON PIG HEART SUCCINYL-COA SYNTHETASE - THE EXTINCTION COEFFICIENT AND AMINO ACID COMPOSITION

A. Introduction

An accurate determination of the concentration of pig heart succinyl-CoA synthetase used in kinetic or physical studies depends upon having a reliable value for the ultra-violet extinction coefficient. Since two very different values for the extinction coefficient of the pig heart enzyme have appeared in the literature ($E_{1\text{ cm}}^{0.1\%}$ at 280 nm=0.35 (65) or 0.90 (66)), it seemed necessary to reevaluate this parameter. The protein-dye binding method of Bradford (69) has proven to be a convenient and accurate means of determining the protein concentration of the *E. coli* enzyme in dilute solutions. It was therefore used to check the value of the extinction coefficient for the pig heart enzyme.

The amino acid compositions of native *E. coli* succinyl-CoA synthetase and its isolated subunits have been presented (54). Succinyl-CoA synthetase has been purified recently from rat liver and amino acid analyses revealed significant similarities in 50% of the amino acid residues of the native rat liver and *E. coli* enzymes (75). No reports have appeared on the analyses of pig heart succinyl-CoA synthetase or of its subunits. These compositions are presented here and comparisons are made between the amino acid analyses of the three native enzymes (pig heart, rat

liver and *E. coli*) and the α and β subunits of two of these enzymes (pig heart and *E. coli*). In order to determine the relatedness of the proteins, an index that is commonly used for concisely expressing the amount of difference between compositions (107,108) is calculated.

B. Materials and Methods

1. Determination of the extinction coefficient

The protein concentration of a solution of purified pig heart succinyl-CoA synthetase was determined by the protein-dye binding method of Bradford (69) using *E. coli* succinyl-CoA synthetase as the standard.

2. Amino acid analysis

The α and β subunits of the pig heart enzyme were prepared by gel filtration according to the methods described by Pearson and Bridger (54). Samples of the native enzyme and its subunits were dialyzed extensively against distilled water and lyophilized. The residues were re-dissolved in constant boiling HCl containing 0.1% phenol and duplicate samples were hydrolyzed *in vacuo* at 110° for 24, 48 and 72 hours. The samples were then evaporated to dryness and analyzed on a Beckman model 120C amino acid analyzer. The values for isoleucine, leucine and valine were taken from the analysis of the 72-h sample. Serine, threonine and methionine were estimated by extrapolation to zero hydrolysis time. For purposes of calculation, the molecular weights of 34,500 (α) and 42,500 (β) were used for the

subunits of the pig heart enzyme. These molecular weights were estimated from polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (49).

C. Results

1. The extinction coefficient

A value of 0.35 was obtained for the extinction coefficient ($E_{1\text{ cm}}^{0.1\%}$) at 280 nm of pig heart succinyl-CoA synthetase using either the normal assay or micro-assay method of Bradford (69). This is to be compared with the extinction coefficient of 0.5 for both the rat liver (75) and *E. coli* (48) enzymes.

2. The amino acid compositions

The results of amino acid analyses of native pig heart succinyl-CoA synthetase and its separated subunits are presented in Table 2. It can be seen that the composition of the native enzyme is in good agreement with that calculated for an $\alpha\beta$ dimer from the compositions of the isolated subunits. As was the case with the *E. coli* enzyme (54), the data rule out the possibility that the smaller (α) subunit is a proteolytic derivative of the larger one (β) since certain residues (e.g. threonine) are present in larger amounts in the α subunit.

In Table 3 the amino acid compositions of the rat liver (75) and *E. coli* (54) enzymes are included along with the composition of pig heart succinyl-CoA synthetase. For purposes of comparison, the molecular weight of the *E. coli*

Table 2

Amino acid compositions of pig heart succinyl-CoA synthetase and of its subunits.

Amino acid	Native enzyme	α -subunit	β -subunit	Calculated for $\alpha\beta$ structure
	residues/77,000 M.W.	residues/34,500 M.W.	residues/42,500 M.W.	
Lysine	59.3	25.1	33.8	59
Histidine	12.3	9.8	3.1	13
Arginine	24.4	10.7	10.0	21
Aspartic acid	75.2	22.4	48.4	71
Threonine	37.3	25.8	16.3	42
Serine	30.7	14.2	15.4	30
Glutamic acid	89.3	35.6	50.7	86
Proline	31.7	20.7	15.3	36
Glycine	78.2	44.9	34.8	80
Alanine	70.3	29.6	43.1	73
Cysteine	N.D. ^a	N.D.	N.D.	
Valine	50.0	22.7	30.7	53
Methionine	11.7	6.2	6.9	13
Isoleucine	44.5	22.7	25.0	48
Leucine	63.5	23.5	38.9	62
Tyrosine	12.8	5.5	6.7	12
Phenylalanine	27.6	10.3	15.6	26
Tryptophan	N.D.	N.D.	N.D.	

^a N.D., not determined.

The amino acid compositions of succinyl-CoA synthetase from three sources.

Amino Acid	Pig heart	Rat liver ^b	E. coli ^c
	residues/ 77,000 M.W.	residues/ 80,000 M.W.	residues/ 68,000 M.W.
Lysine	59	55	44
Histidine	12	11	10
Arginine	24	20	21
Aspartic acid	75	72	52
Threonine	37	36	39
Serine	31	45	20
Glutamic acid	89	112	68
Proline	32	29	27
Glycine	78	85	80
Alanine	70	76	72
Cysteine	N.D. ^a	22	12
Valine	50	47	58
Methionine	12	15	13
Isoleucine	45	55	47
Leucine	64	58	52
Tyrosine	13	15	11
Phenylalanine	28	29	19
Tryptophan	N.D.	2	4
Total:	719	784	649

^a
N.D., not determined

^bdata from Ball and Nishimura (75)

^cBased on the data of Pearson and Bridger (54)

enzyme is taken to be 68,000 (the dimeric molecular weight). There appear to be similarities between all three enzymes. An index commonly used for expressing the amount of difference between the amino acid composition of proteins (108) was calculated to test their relatedness.

$$S\Delta n = 1/2 \sum (n_{iA} - n_{iB})^2 - 0.035 (N_A - N_B)^2 + 0.535 |N_A - N_B|$$

where n_{iA} and n_{iB} are the numbers of amino acid residues of the i th type in protein A and B, and N_A and N_B are the total number of residues in A and B. The so-called 'strong' test predicts that the two proteins are related in their sequence only when $S\Delta n$ is less than $0.42N$, where N equals the total number of residues in the smaller of the two proteins. In the less demanding 'weak' test $S\Delta n$ is taken as significant if it is less than $0.93 N$. In a comparison of 163 pairs of proteins the 'strong' test identified only 7 of the 19 indisputably related pairs. However, the 'weak' test detected genuine similarity in 22 out of 23 cases but incorrectly indicated similarity in 16 (107).

The amino acid analyses of the three succinyl-CoA synthetases in Table 3 were compared using the above mentioned index and the calculated values of $S\Delta n$, $0.42 N$ and $0.93 N$ (in that order) are shown below for each combination:

pig heart-rat liver: 398, 302, 669

pig heart-*E. coli*: 697, 273, 604

rat liver-*E. coli*: 1226, 273, 604

It can be seen that the value of $S_{\Delta n}$ is greater than 0.42 N but less than 0.93 N for the pig heart - rat liver enzyme pair. According to the theory of Cornish-Bowden (108), this indicates that the enzymes from these two mammalian species probably have similar sequences since the 'weak' test is passed. However, neither the pig heart or rat liver succinyl-CoA synthetase appear to be related to the enzyme from *E. coli* by these criteria since $S_{\Delta n}$ is greater than 0.93 N for both comparisons.

The amino acid compositions of the separated subunits of the pig heart and *E. coli* enzymes are displayed in Table 4. Calculations were done comparing the two α -subunits and the values of $S_{\Delta n}$, 0.42 N and 0.93 N were determined to be 116, 124 and 276 respectively. This indicates that the two α -subunits are almost certainly related since $S_{\Delta n}$ is less than 0.42 N, i.e. the 'strong' test is passed. When the same analysis was done on the β -subunits, $S_{\Delta n}$, 0.42 N and 0.93 N were calculated as 275, 152 and 338. Therefore the β -subunits from pig heart and *E. coli* succinyl-CoA synthetase may be similar (the 'weak' test showed positive results).

D. Discussion

The ultraviolet extinction coefficient ($E_{1\text{ cm}}^{0.1\%}$ at 280 nm) of pig heart succinyl-CoA synthetase was found to be 0.35 when reevaluated using a protein-dye binding method. This value is in good agreement with that previously

Table 4

A comparison between the amino acid compositions of the subunits of pig heart and *E. coli* succinyl-CoA synthetase.

Amino acid	Pig heart α -subunit (A)	α -subunit E. coli (B)	α -subunit E. coli ^b (B)	(A)-(B)	β -subunit Pig heart (C)	β -subunit E. coli ^b (D)	(C)-(D)
	residues/ 34,500 M.W.	residues/ 29,500 M.W.	residues/ 29,500 M.W.		residues/ 42,500 M.W.	residues/ 38,500 M.W.	
Lysine	25.1	21.4	21.4	+3.7	33.8	22.6	+11.2
Histidine	9.8	4.8	4.8	+5.0	3.1	4.0	-0.9
Arginine	10.7	6.0	6.0	+4.7	10.0	15.3	-5.3
Aspartic acid	22.4	17.3	17.3	+5.1	48.4	34.2	+14.1
Threonine	25.8	25.2	25.2	+0.6	16.3	15.6	+0.7
Serine	14.2	12.5	12.5	+1.7	15.4	9.5	+5.9
Glutamic acid	35.6	28.7	28.7	+6.9	50.7	44.1	+6.6
Proline	20.7	15.7	15.7	+5.0	15.3	13.0	+2.3
Glycine	44.9	42.9	42.9	+2.0	34.8	42.7	-7.9
Alanine	29.6	30.0	30.0	-0.4	43.1	43.1	0
Cysteine	N.D. ^a	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Valine	22.7	26.4	26.4	-3.7	30.7	37.0	-6.3
Methionine	6.2	6.6	6.6	-0.4	6.9	7.6	-0.7
Isoleucine	22.7	28.8	28.8	-6.1	25.0	21.3	+3.7
Leucine	23.5	16.4	16.4	+7.1	38.9	36.1	+2.8
Tyrosine	5.5	6.3	6.3	-0.8	6.7	6.7	0
Phenylalanine	10.3	8.4	8.4	1.9	15.6	10.5	+5.1
Tryptophan	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Total:	330.0	297.0	297.0		395.0	363.0	

^a N.D., not determined

^b data from Pearson and Bridger (54)

determined by Murakami and Nirhimura (67). The extinction coefficient of 0.90 used by Cha (66) appears to be incorrect.

When the amino acid analyses of the three native succinyl-CoA synthetases (pig heart, rat liver and *E. coli*) were compared with the aid of a commonly-used index, it was found that the two mammalian enzymes may have similar primary sequences while the *E. coli* enzyme did not appear by this test to be related to either of those enzymes. It is not at all surprising that the two eukaryotic enzymes are more closely related to each other than they are to their prokaryotic counterpart.

However, when the amino acid compositions of the separated subunits of the pig heart and *E. coli* enzyme were compared, it was found, according to the criteria developed by Cornish-Bowden (107), that the two α -subunits (one eukaryotic and one prokaryotic) were certainly related. When the number of amino acid residues of each type in the smaller *E. coli* α -subunit are subtracted from the number of residues in the larger pig heart α -subunit some interesting trends are revealed. For example, the number of non-polar amino acid residues (alanine, valine, leucine, isoleucine, proline, phenylalanine and methionine) increases by only 3.4 in the larger pig heart α -subunit. Similarly, there is a small increase (3.5) in the number of amino acid residues with polar uncharged side chains (glycine, serine, threonine, tyrosine). However, the pig heart α -subunit

contains an additional 25.4 charged amino acids (aspartic acid, glutamic acid, lysine, arginine, histidine). It appears then that the increase in size between the pig heart and *E. coli* α -subunit is due largely to the addition of charged amino acids. Although the two β -subunits are not as closely related according to the index, the increased size of the pig heart β -subunit also results from the addition of charged groups, i.e. there are 5.6 additional nonpolar and uncharged polar amino acid residues but 25.7 additional amino acids having charged side chains.

The reason for the index giving positive correlations between the isolated subunits of the pig heart and *E. coli* enzymes, while indicating no similarities between the two enzymes when they are compared as a whole, may be due to the size difference between the enzymes. Comparisons of the compositions of proteins of very different lengths are difficult to interpret. However, when the separated subunits are analyzed this size difference is reduced.

It is intriguing that the α -subunit in pig heart succinyl-CoA synthetase is closely related to the α -subunit in the *E. coli* enzyme, yet contains an additional 25 charged amino acids, especially with regard to certain observations on the nature of subunit contacts in other oligomeric proteins. There is evidence that many protein-protein interactions involve polypeptide chain termini. For example, the C-terminal plays an important role for the polypeptides of α -chymotrypsin (109) and the subunits of hemoglobin (110),

and the N-terminal residues may be involved in polypeptide interactions in procarboxypeptidase A (111). Particularly interesting conclusions have been drawn from comparisons of cytoplasmic malate dehydrogenase and lactate dehydrogenase. Malate dehydrogenase is comprised of two subunits and is conformationally highly homologous with the tetrameric lactate dehydrogenase. Lactate dehydrogenase is a tetramer of four identical subunits while malate dehydrogenase is a dimer, apparently only because of an additional 20 amino acid residues at the N-terminus of lactate dehydrogenase (112). With this example in mind, it is suggested that pig heart succinyl-CoA synthetase exists predominantly as a dimer (while the *E. coli* enzyme lies far towards the tetramer in the dimer-tetramer equilibrium) simply because of an additional group of highly charged residues being added to the end of the polypeptide chain in the α -subunit. As with most conclusions based on a comparison of amino acid compositions, this suggestion must of course be regarded to be highly speculative, and can only be confirmed through a determination of the primary sequence and X-ray structure of both proteins.

VIII. CONCLUSIONS

The majority of the work reported in this thesis was directed towards answering two essential questions: 'What are the functions of the two types of subunits in succinyl-CoA synthetase?' and 'Why does the *E. coli* enzyme have two potential active sites and pig heart enzyme only one?'. It was hoped that these studies would add to the understanding of structure-function relationships in other oligomeric enzymes.

A. *Why two kinds of subunits?*

Affinity chromatography of the separated subunits of the *E. coli* enzyme showed that the isolated β subunit is capable of binding CoA. Recombination studies involving NBD-Cl modified subunits further demonstrated that both subunit types are important for enzyme activity. These results are compatible with the previously proposed picture of catalysis with functional roles for both subunits. The reaction may be visualized as beginning on the α subunit with transfer of the terminal phosphoryl group of ATP to a histidine residue of that subunit. The subsequent transfer of the phosphoryl group to succinate and its displacement by CoA to yield succinyl-CoA and Pi is thought to occur on the β subunit. Other oligomeric enzyme systems have been described in which separate partial reactions occur within the domains of different subunits: for example, tryptophan synthase (4), carbamyl phosphate synthetase (113), and

transcarboxylase (114).

B. Why two copies of each subunit per enzyme molecule?

The rationale for the tetrameric structure of the *E. coli* enzyme remains less than clear. Bild *et al.* (29) had proposed that the negative cooperativity of phosphorylation shown by the *E. coli* enzyme reflects the operation of catalytic cooperativity between active sites and that this could be an advantage that nature gains from the tetrameric enzyme structure. Attempts to validate this hypothesis through a study of the oxygen exchange patterns of hybrid enzyme molecules were complicated by the finding that the oxygen exchange kinetics varied as a function of enzyme concentration. When this effect was investigated in more detail it became apparent that a model not involving catalytic cooperativity, but proposing a dimer-tetramer equilibrium for both the *E. coli* and pig heart enzyme, could adequately explain all the oxygen exchange data. Initial velocity kinetics and physical studies provided convincing evidence for this equilibrium. The initial rate analysis revealed that the form of succinyl-CoA synthetase predominant at low enzyme concentrations (the dimer) has an increased specific activity. Thus, although the operation of catalytic cooperativity between active sites in the intact tetramer can not be excluded by these experiments, it is not apparent what selective advantage the enzyme would gain from these interactions since the independent active site appears to

operate with increased catalytic efficiency.

In view of these results, it seems appropriate to survey the status of the concept of catalytic cooperativity, it having been exposed to the test of time in other enzyme systems. Grubmeyer and Penefsky (115) have recently reported an interesting study that described the interactions between two catalytic sites on beef heart mitochondrial ATPase (F_1) during hydrolysis of the ATP analog TNP-ATP. It was shown that hydrolysis of substrate bound in one site is accelerated when TNP-ATP or other nucleotides are allowed to bind to the second site. This, together with the studies of Boyer and colleagues on the oxygen exchange catalyzed by membrane-bound and soluble F_1 (23,26,27) provide convincing evidence for the operation of catalytic cooperativity in this system. The characteristic stimulation of exchange at low substrate concentration was also observed for the CF_1 ATPase of chloroplasts that catalyzes photophosphorylation (24). Fast-kinetic evidence for an activating effect of ATP on the Ca^{2+} transport of sarcoplasmic reticulum ATPase was interpreted as indicating catalytic cooperativity (116). However, a study of the vanadate-trapped state of the (Na,K)-ATPase provided evidence against the interacting nucleotide site model (117). The operation of catalytic cooperativity has also been proposed for several non-ATPase enzymes, most notably malate dehydrogenase and alkaline phosphatase. Recent modification studies on malate dehydrogenase have shown that the hybrid molecule

contributes half of the enzymatic activity of the native dimer (118), a result not in harmony with the operation of inter-site cooperativity. Two kinetic studies on alkaline phosphatase, one involving alternative substrate and product inhibition studies and catalytic rate constant measurements (31) and the other using a transient kinetic analysis (119) have led to the proposal of a catalytic cycle which is different from the catalytic cooperative model. Also, it has been reported that *E. coli* alkaline phosphatase does not exhibit the characteristic substrate concentration-dependent exchange pattern (89). Thus it appears that the information available at this time leans favourably towards the concept of catalytic cooperativity as proposed for the membrane-bound ATPases but away from the possibility of its operation in malate dehydrogenase and alkaline phosphatase.

Although there is no evidence in the literature that catalytic cooperativity acts to accelerate rate-limiting steps in the pathway of the succinyl-CoA synthetase reaction, there are indications of site-site interactions in the *E. coli* enzyme tetramer. Hans Vogel (in our laboratory) has observed, using ^{31}P NMR, that the addition of ATP to a concentrated solution of previously phosphorylated *E. coli* enzyme leads to the appearance of the resonance characteristic of the succinyl phosphate intermediate. This implies that ATP binding or phosphorylation at one active site triggers phosphoryl transfer from histidine to succinate at the other site. A consideration of the effect

of CoA on intermediate steps in the reaction makes it seem unlikely that this ATP-modulation of succinyl phosphate formation relates to the ATP-dependent exchange catalyzed at high enzyme concentrations. That is, since CoA also affects the rate of succinyl phosphate production (41), an example of an effect known by the term 'substrate synergism', then the relative rate of oxygen exchange should be both CoA- and ATP-dependent. However, this was not found to be the case (29). It appears that the proposed dimer-tetramer equilibrium may still be the best explanation for the apparent ATP modulation of the exchange reaction.

Be that as it may, it has been shown that *E. coli* succinyl-CoA synthetase possesses at least the capacity for alternating sites cooperativity in catalysis. Part of my early work involved the evaluation of possible methods for the separation of the phospho- and dephospho- α subunit. Although we were unsuccessful in separating α from α -P, this study led to the development of a selective chromatographic procedure for the separation of the phospho- and dephosphopeptides derived from the α subunits. This enabled us to show, with the aid of a hybrid enzyme containing one ^{35}S -labelled α subunit (dephosphorylated) and one nonradioactive α subunit (phosphorylated), that both active sites are available for phosphorylation (61), and that the enzyme therefore has the capacity for alternating sites cooperativity. This publication is reproduced in Appendix 2.

C. The dimer-tetramer equilibrium of succinyl-CoA synthetase

The oxygen exchange and initial rate kinetics of both the *E. coli* and pig heart enzyme revealed that several parameters (the relative rate of oxygen exchange, V_{\max} and K_m) are dependent upon the enzyme concentration. This behaviour is characteristic of a self-associating system in which the catalytic activity of the enzyme varies with the degree of association. Many oligomeric enzymes are known to undergo association-dissociation reactions and in some cases the specific activity of the subunit has been shown to depend on the state of association of the enzyme. Glycer-aldehyde-3-phosphate dehydrogenase is an example that resembles succinyl-CoA synthetase in that the specific activity of the enzyme increases with dissociation (120). Carbamyl phosphate synthetase (121), arginine decarboxylase (122) and β -isopropylmalate dehydrogenase (123) are a few more examples of enzymes with concentration-dependent kinetic properties. It appears then that *E. coli* and pig heart succinyl-CoA synthetase share this property with several other enzymes. It is unlikely that the protein concentration effects could be attributable to a small molecule contaminant in the enzyme preparation since the enzyme was subjected to gel filtration immediately prior to all experiments, and also since ^1H and ^{31}P NMR have revealed no unexpected resonances in the spectra of concentrated enzyme solutions.

Taking previously published inferences into consideration, it is not surprising that succinyl-CoA synthetase should undergo a dimer-tetramer equilibrium. Cross-linking of the *E. coli* enzyme with diiminoesters was shown to result principally in the $\alpha\beta$ dimer, and reaction with dimaleimides yielded solely the $\alpha\beta$ dimer (124). These results suggest an architectural arrangement for the enzyme molecule where there is intimate association of the α and β monomers and loose contact regions between the two $\alpha\beta$ dimers. Hence, the structure of the *E. coli* tetramer was most appropriately described as a 'dimer of dimers'. This structure is consistent with the proposed ability of the *E. coli* tetramer to dissociate easily to the dimeric state, and yet to retain this active conformation without dissociating further to inactive monomers. Sedimentation equilibrium studies on the *E. coli* enzyme had also hinted towards the dissociation of the enzyme to dimers at low protein concentrations (48). Furthermore, it has been suggested that malate thiokinase, an enzyme with catalytic properties and a subunit structure similar to succinyl-CoA synthetase, has an $\alpha_2\beta_2$ - $\alpha_4\beta_4$ equilibrium (125).

The proposed dimer-tetramer equilibrium may explain a curious point concerning the specific activity of the purified *E. coli* enzyme. It has been reported that apparently homogeneous enzyme preparations can display specific activities ranging from 20-40 units/mg (2). If the degree of association varied depending on the assay

conditions used (i.e. high salt versus low salt, and the concentration of enzyme used in the assay) then according to the model proposed here, the catalytic activity of the enzyme would be expected to vary. Thus, to compare the activities of different preparations one should keep the assay conditions relatively constant. Another matter of contention that may be related to the state of aggregation of the enzyme is the stoichiometry of phosphorylation of *E. coli* succinyl-CoA synthetase. Nishimura and colleagues have consistently reported a stoichiometry of phosphorylation approaching two (58,59) while our laboratory and others have repeatedly observed an incorporation of one phosphoryl group per mole of enzyme (44,18,65). If the enzyme was in its tetrameric state during phosphorylation, negative cooperativity between sites would limit the phosphorylation to one group per tetramer. However, these sites would become independent upon dissociation to dimers and the stoichiometry of phosphorylation would approach two. Perhaps the different stoichiometries reported by different groups can be traced to experimental details that affect the aggregation state of the enzyme. One of my earlier projects involved a search for an enzyme concentration effect on the stoichiometry of phosphorylation of *E. coli* succinyl-CoA synthetase. Unfortunately, the experiments were hindered by technical difficulties resulting from the use of dilute protein solutions and radioactive ATP of high specific activity.

D. Physiological significance of the dimer-tetramer equilibrium

The remaining problem is to deduce what the oligomeric state of succinyl-CoA synthetase is within the cell. Such deduction would have to be based upon knowledge of the intracellular concentration of the enzyme, the presence and concentration of ligands that might increase or decrease the association, and knowledge of other macromolecules or organelles that influence the association. The dimer-tetramer equilibrium of *E. coli* succinyl-CoA synthetase might well have important implications for metabolic regulation since the kinetic properties of the enzyme are influenced by the oligomeric state. In relation to this, Ottaway (126) has recently proposed that succinyl-CoA synthetase may be rate-limiting within the Citric Acid Cycle. From a structural point of view, association may promote stability under physiological conditions, i.e. it may influence the turnover of the enzyme.

Calculations of the intracellular concentration of *E. coli* succinyl-CoA synthetase range around 1 to 5 mg/ml. The kinetic and physical studies reported in this thesis have indicated that the enzyme is predominantly tetrameric in this protein concentration range. Sedimentation equilibrium showed that relatively low ionic strengths and the presence of Pi (conditions found within the cell) promote association of the enzyme. The recently described 'crowding' effect (120) (a phenomenon caused by the space-filling properties

of high concentrations of unrelated globular proteins) would also promote formation of the tetramer. Beyond this, it is difficult to extrapolate the solution properties of *E. coli* succinyl-CoA synthetase to the *in vivo* state of the enzyme since the effect of other substrates, ligands and macromolecules on the equilibrium is not known. The following discussion of the well-studied example of yeast hexokinase illustrates that the situation is often not straight forward. Calculations of the intracellular concentration of the enzyme, together with the known associating effect of many intracellular conditions (i.e. pH, presence of substrates and activators, etc.) all led to the conclusion that yeast hexokinase would behave as a dimer within the cell. However, recent studies on the cooperativity of ATP with hexokinase in permeabilized 'ghost' cells have shown that the enzyme behaves as if it were monomeric (127). This emphasizes the difficulty of extrapolating results directly to the physiological situation. Because of the extremely high protein concentrations within the mitochondria (56% w/w), it would be even more difficult to deduce the oligomeric state of pig heart succinyl-CoA synthetase *in vivo*. Although the solution studies reported herein revealed that the equilibrium for the enzyme is far towards the dimer, the experimental conditions do not even resemble those within the cell. In fact, protein crystals and their properties may better represent the conditions experienced by the proteins of the mitochondrial matrix (128).

No matter what the oligomeric state of the enzyme is within the cell, it is obvious that the association properties of succinyl-CoA synthetase from various sources differ substantially *in vitro*, i.e. the enzymes from Gram-negative bacteria are predominantly tetrameric in solution while Gram-positive bacteria and all eukaryotic organisms produce 'dimeric' succinyl-CoA synthetases (53). This structural homology between mammalian and Gram-positive bacterial forms of the enzyme may be explained by the endosymbiosis theory for the evolution of the mitochondrion (129). If invasion of a proto-eukaryote by a bacterium with Gram-positive characteristics had led to the evolution of a eukaryotic cell, then the mitochondrial succinyl-CoA synthetase would be expected to retain structural similarities to the Gram-positive bacterial form of the enzyme.

In summary, the studies reported in this dissertation show that succinyl-CoA synthetase is a predictably complex enzyme and that it exhibits many of the characteristics of the 'worst' case of a cooperative enzyme as defined by Neet (130), i.e., it appears to have (a) a dimer-tetramer equilibrium; (b) site-site interactions within the tetramer; (c) a different number of ligand binding sites per subunit in the two species; (d) a different catalytic rate constant in each of the oligomeric species; (e) three substrates, so that the cooperativity of one ligand may depend on another substrate; and (f) a possible hysteretic transition. It is difficult to say whether this type of behaviour is the

culmination of eons of evolution in developing the optimal enzyme for a particular function *OR* whether the kinetic and structural properties revealed may be accidents of evolution which, having no physiological advantage and also causing no harm, have not been excised.

IX. Bibliography

1. Sanwal, B.D. (1970) *Bacteriol. Rev.* 34, 20-39
2. Bridger, W.A. (1974) in *The Enzymes* (Boyer, P., ed.) Vol. 10, 3rd Ed., pp. 581-606, Academic Press, New York
3. Darnall, D.W., and Klotz, I.M. (1975) *Arch. Biochem. Biophys.* 166, 651-682
4. Miles, E.W. (1979) *Adv. Enzymol.* 49, 127-186
5. Evans, P.R., and Hudson, P.J. (1979) *Nature (Lond.)* 279, 500-504
6. Chan, W.W.-C. (1976) *Can. J. Biochem.* 54, 521-528
7. Miles, E.W., and Higgins, W. (1978) *J. Biol. Chem.* 253, 6266-6269
8. Monod, J., Changeux, J.P., and Jacob, F. *J. Mol. Biol.* 6, 306-329
9. Kantrowitz, E.R., Pastra-Landis, S.C., and Lipscomb, W.N. (1980) *T.I.B.S.* 5, 150-153
10. Fletterick, R.J., and Madsen, N.B. (1980) *Annu. Rev. Biochem.* 49, 31-61
11. Uyeda, K. (1979) *Adv. Enzymol.* 48, 193-244
12. Monod, J., Wyman, J., and Changeux, J.P. (1965) *J. Mol. Biol.* 12, 88-118
13. Chen, A.K., et al. (1974) *Biochemistry* 13, 654-661
14. Wyman, A., and Paulus, H. (1975) *J. Biol. Chem.* 250, 3897-3903
15. Levitzki, A., and Koshland, D.E. (1976) *Curr. Top. Cell. Regul.* 10, 1-40
16. Malhotra, O.P., Bernhard, S.A., and Seydoux, F. (1981)

Biochimie 63, 131-141

17. Coleman, J.E., and Chlebowski, J.F. (1979) in *Advances in Inorganic Biochemistry* (Eichhorn, G.L., and Marzilli, L.G., eds.) pp. 1-66 Elsevier North Holland, Inc., New York
18. Moffet, F.J., Wang, T., and Bridger, W.A. (1972) *J. Biol. Chem.* 247, 8139-8144
19. Harada, K., and Wolfe, R.G. (1968) *J. Biol. Chem.* 243, 4131-4137
20. Lazdunski, M., et al. (1971) *Eur. J. Biochem.* 20, 124-139
21. Kayalar, C., Rosing, J., and Boyer, P.D. (1977) *J. Biol. Chem.* 252, 2486-2491
22. Wimmer, M.J., and Rose, I.A. (1977) *J. Biol. Chem.* 252, 6769-6775
23. Hackney, D.D., and Boyer, P.D. (1978) *J. Biol. Chem.* 253, 3164-3170
24. Hackney, D.D., Rosen, G., and Boyer, P.D. (1979) *Proc. Nat. Acad. Sci. U.S.A.* 76, 3646-3650
25. Russo, J.A., Lamos, C.M., and Mitchell, R.A. (1978) *Biochemistry* 17, 473-480
26. Choate, G.L., Hutton, R.L., and Boyer, P.D. (1979) *J. Biol. Chem.* 254, 286 - 290
27. Hutton, R.L., and Boyer, P.D. (1979) *J. Biol. Chem.* 254, 9990-9993
28. Bild, G.S., and Boyer, P.D. (1980) *Biochemistry* 19, 5774-5781

29. Bild, G.S., Janson, C.A., and Boyer, P.D. (1980) *J. Biol. Chem.* 255, 8109-8115
30. Gutfreud, H. (1975) *Prog. Biophys. Mol. Biol.* 29, 161-195
31. Bale, J.R., Chock, P.B., and Huang, C.Y. (1980) *J. Biol. Chem.* 255, 8424-8430
32. Green, N.M. (1971) in *Protein-Protein Interactions* (Jaenicke, R. and Helmreich, E., eds.) pp. 209 Springer-Verlag, New York
33. Kaufman, S. (1955) *J. Biol. Chem.* 216, 153-164
34. Upper, C.D. (1964) Ph.D. Thesis, Univ. of Illinois, Urbana
35. Kreil, G., and Boyer, P.D. (1964) *Biochem. Biophys. Res. Commun.* 16, 551-555
36. Bridger, W.A., Millen, W.A., and Boyer, P.D. (1968) *Biochemistry* 7, 3608-3616
37. Hager, L.P. (1957) *J. Amer. Chem. Soc.* 79 4864-4866
38. Cohn, M. (1959) *J. Cell. Comp. Physiol.* 54 17-31
39. Nishimura, J.S., and Meister, A. (1965) *Biochemistry* 4 1457-1462
40. Robinson, J.L., Benson, R.W., and Boyer, P.D. (1969) *Biochemistry* 8, 2503-2508
41. Grinnell, F.L., and Nishimura, J.S. (1969) *Biochemistry* 8, 568-574
42. Hildebrand, J.G., and Spector, L.B. (1969) *J. Biol. Chem.* 244, 2606-2613
43. Cha, S., Cha, C.-J.M., and Parks, R.E. (1967) *J. Biol.*

Chem. 242, 2582-2592

44. Ramaley, R.F., Bridger, W.A., Moyer, R.W., and Boyer, P.D. (1967) *J. Biol. Chem.* 242 4287-4298
45. Grinnel, F.L., and Nishimura, J.S. (1970) *Biochim. Biophys. Acta* 212, 150-157
46. Leitzmann, C., Wu, J.-Y., and Boyer, P.D. (1970) *Biochemistry* 9, 2338-2346
47. Bridger, W.A. (1971) *Biochem. Biophys. Res. Commun.* 42, 948-954
48. Krebs, A., and Bridger, W.A. (1974) *Can. J. Biochem.* 52, 594-598
49. Brownie, E.R., and Bridger, W.A. (1972) *Can. J. Biochem.* 50, 719-724
50. Cha, S., Cha, C.-J.M., and Parks, R.E. (1967) *J. Biol. Chem.* 242, 2577-2581
51. Baccanari, D.P., and Cha, S. (1973) *J. Biol. Chem.* 248, 15-24
52. Kelly, C.J., and Cha, S., (1977) *Arch. Biochem. Biophys.* 178, 208-217
53. Weitzman, P.D.J., and Kinghorn, H.A. (1978) *FEBS Lett.* 88, 255-258
54. Pearson, P.H., and Bridger, W.A. (1975) *J. Biol. Chem.* 250, 4451-4455
55. Pearson, P.H., and Bridger, W.A. (1975) *J. Biol. Chem.* 250, 8524-8529.
56. Collier, G.E., and Nishimura, J.S. (1979) *J. Biol. Chem.* 254, 10925-10930

57. Moffet, F.J., Wang, T., and Bridger, W.A. (1972) *J. Biol. Chem.* 247, 8139-8144
58. Grinnell, F.L., and Nishimura, J.S. (1969) *Biochemistry* 8, 562-568
59. Bowman, C.M., and Nishimura, J.S. (1975) *J. Biol. Chem.* 250, 5609-5613
60. Lam, Y., Bridger, W.A., and Kotowycz, G. (1976) *Biochemistry* 15, 4742-4748
61. Wolodko, W.T., O'Connor, M.D. and Bridger, W.A. (1981) *Proc. Nat. Acad. Sci. U.S.A.* 78, 2140-2144
62. Riordan, J.F., McElvany, K.D., and Borders, C.L., Jr. (1977) *Science* 195, 884-886
63. Takahashi, K. (1968) *J. Biol. Chem.* 243, 6171-6179
64. Birkett, D.J. et al. (1970) *FEBS Lett.* 6, 346-348
65. Wolodko, W.T., Brownie, E.R., and Bridger, W.A. (1980) *J. Bacteriol.* 143, 231-237
66. Cha, S. (1969) *Methods Enzymol.* 13, 62-69
67. Murakami, Y., and Nishimura, J.S. (1974) *Biochim. Biophys. Acta* 336, 252-263
68. Bridger, W.A., Ramaley, R.F., and Boyer, P.D. (1969) *Methods Enzymol.* 13, 70-75
69. Bradford, M.M. (1976) *Analytical Biochem.* 72, 248-254
70. Ray, W.J., Jr., and Koshland, D.E., Jr. (1961) *J. Biol. Chem.* 236, 1973-1978
71. Birkett, D.J. et al. (1971) *Eur. J. Biochem.* 20, 494-508
72. Birkett, D.J. et al. (1970) *FEBS Lett.* 6, 346-348

73. Collier, G.E., and Nishimura, J.S. (1978) *J. Biol. Chem.* 253, 4938-4943
74. Nishimura, J.S., et al. (1980) *Biochem. Int.* 1, 339-345
75. Ball, D.J., and Nishimura, J.S. (1980) *J. Biol. Chem.* 255, 10805-10812
76. Matula, J.M., and Nishimura, J.S. (1978) *Int. J. Biochem.* 9, 213-215
77. Nishimura, J.S., Kenyon, G.L., and Smith, D.J. (1975) *Arch. Biochem. Biophys.* 170, 461-467
78. Nishimura, J.S., Mitchell, T., and Matula, J.M. (1976) *Biochem. Biophys. Res. Commun.* 69, 1057-1064
79. Risley, J.M., and Van Etten, R.L. (1978) *J. Labelled Compd. Radiopharm.* 15, 533-537
80. Hackney, D.D., Stempel, K.E., and Boyer, P.D. (1980) *Methods Enzymol.* 64, 60-83
81. Kaufman, S. (1955) *Methods Enzymol.* 1, 714-722
82. Lipmann, F., and Tuttle, L.C. (1945) *J. Biol. Chem.* 158, 505-519
83. Cohn, M., and Hu, A. (1978) *Proc. Nat. Acad. Sci. U.S.A.* 75, 200-203
84. Kornberg, A., and Horecker, B.L. (1953) in *Biochemical Preparations* (Snell, E.E., ed.) Vol. 3, pp.23, Wiley and Sons, New York
85. Bridger, W.A. (1981) *Can. J. Biochem.* 59, 1-8
86. Pugh, E.M., and Winslow, G.H. (1966) *The Analysis of Physical Measurements* pp. 119-136, Addison-Wesley, Reading, Mass.

87. Aboderin, A.A., Boedefeld, E., and Luisi, P.L. (1973) *Biochim. Biophys. Acta* 328, 20-30
88. Ferguson, S.J., Lloyd, W.J., and Radda, G.K. (1975) *Eur. J. Biochem.* 54, 127-133
89. Hackney, D.D. (1980) *J. Biol. Chem.* 255, 5320-5328
90. Hackney, D.D., and Boyer, P.D. (1978) *Proc. Nat. Acad. Sci. U.S.A.* 76, 3646-3650
91. Midelfort, C.F. (1981) *Proc. Nat. Acad. Sci. U.S.A.* 78, 2067-2071
92. Shukla, K.K., et al. (1980) *J. Biol. Chem.* 255, 11344-11350
93. Sleep, J.A., Hackney, D.D., and Boyer, P.D. (1980) *J. Biol. Chem.* 255, 4094-4099
94. Sleep, J.A., Hackney, D.D., and Boyer, P.D. (1978) *J. Biol. Chem.* 253, 5235-5238
95. Damodaran, S., and Kinsella, J.E. (1981) *J. Biol. Chem.* 256, 3394-3398
96. Herskouits, T.T., et al. (1981) *Biochemistry* 20, 2580-2587
97. Bush, L.P. (1969) *Plant Phys.* 44, 347-350
98. Wider de Xifra, E.A., et al. (1980) *Int. J. Biochem.* 12, 717-719
99. Nishimura, J.S., Mitchell, T., and Grinnell, F. (1973) *J. Biol. Chem.* 248, 743-748
100. Osterlund, B.R., et al. (1980) *Arch. Biochem. Biophys.* 205, 489-498
101. Schachman, H.K. (1957) *Methods Enzymol.* 4, 32-103

102. Shill, J.P., Peters, B.A., and Neet, K.E. (1974) *Biochemistry* 13, 3864-3871
103. Harrington, W.F., and Kegeles, G. (1973) *Methods Enzymol.* 27, 306-345
104. Rippa, M., et al. (1981) *Biochem. J.* 197, 747-749
105. Sund, H., Markau, K., Koberstein, R. (1975) in *Biological Macromolecules, Subunits in Biological Systems* (Fasman, G.D., and Timasheff, S.N., eds.) 7 pp. 225-287, Dekker, New York
106. Frieden, C. (1981) in *Protein-Protein Interactions* (Frieden, C., and Nichol, L.W., eds.) pp. 289-314, Wiley and Sons, Toronto
107. Cornish-Bowden, A. (1980) *Analytical Biochem.* 105, 233-238
108. Cornish-Bowden, A. (1979) *J. Theor. Biol.* 76, 369-386
109. Gladner, J.A., and Neurath, H. (1954) *J. Biol. Chem.* 206, 911-924
110. Perutz, M.F. (1970) *Nature* (London) 228, 726-734
111. Brown, J.R., et al. (1963) *Biochemistry* 2, 867-876
112. Rossman, M.G. et al. (1975) in *The Enzymes* (Boyer, P., ed.) Vol. 11, 3rdEd., pp. 61-102, Academic Press, New York
113. Trotta, P.P., Pinkus, L.M., Haschemeyer, R.M., and Meister, A. (1974) *J. Biol. Chem.* 249, 492-499
114. Chuang, M., Ahmad, F., Jacobson, B., and Wood, H.G. (1975) *Biochemistry* 14, 1611-1619
115. Grubmeyer, C., and Penefsky, H.S. (1981) *J. Biol. Chem.*

256, 3728-3734

116. Verjovski-Almeida, S., and Inesi, G. (1979) *J. Biol. Chem.* 254, 18-21
117. Smith, R.L., Zinn, K., and Cantley, L.C. (1980) *J. Biol. Chem.* 255, 9852-9859
118. Jurgensen, S.R., Harrison, J.H. (1981) *Fed. Proc.* 40, p. 1614
119. Bale, J.R., Chock, P.B., and Huang, C.Y. (1980) *J. Biol. Chem.* 255, 8431-8436
120. Minton, A.P., and Wilf, J. (1981) *Biochemistry* 20, 4821-4826
121. Power, S.G., Meister, A., and Haschemeyer, R.H. (1980) *J. Biol. Chem.* 255, 1554-1558
122. Nowak, S., and Boeker, E.A. (1981) *Arch. Biochem. Biophys.* 207, 110-116
123. Hsu, Y.-P., and Kohlhaw, G.B. (1980) *J. Biol. Chem.* 255, 7255-7260
124. Teherani, J.A., and Nishimura, J.S. (1975) *J. Biol. Chem.* 250, 3883-3890
125. Elwell, M., and Hersh, L.B. (1979) *J. Biol. Chem.* 254, 2434-2438
126. Ottaway, J.H., McClellan, J.A. and Saunderson, C.L. (1981) *Int. J. Biochem.* 13, 401-410
127. Neet, K.E. (1979) *Bull. of Molec. Biol. and Medicine* 4, 101-138
128. Srere, P.A. (1981) *T.I.B.S.* 6, 4-7
129. Margulis, L. (1970) *Origins of Eukaryotic Cells*, Yale

University Press, New Haven

130. Neet, K.E. (1980) *Methods Enzymol.* **64**, 139-192

X. Appendix 1

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1      C
2      C      PROGRAM: MEDIUM PHOSPHATE=WATER EXCHANGE
3      C      CALCULATION OF DISTRIBUTION OF PHOSPHATE SPECIES
4      C
5      C
6      C
7      REAL K, K18
8      DOUBLE PRECISION R, P, OBAR, P10, P11, P20, P21, P22, P30, P31,
9      1 P32, P33, P40, P41, P42, P43, P44
10     C
11     C      INPUT INITIAL DISTRIBUTION
12     C
13     READ(5,7) H00, H10, H20, H30, H40
14     O180 = .025*H10 + .05*H20 + .075*H30 + .1*H40
15     DO 101 J = 1,50
16     READ(5,8,END=200) P, O181
17     C
18     C      CALCULATE OTHER RELATIONSHIPS
19     C
20     X = P/(1.-P)
21     OBAR = 4.*P/(4.-3.*P)
22     OP = OBAR/P
23     R = 4. - 3.*P
24     C
25     C      CALCULATE RATE PARAMETERS
26     C
27     TO = 0.
28     K = 4.*.06931471806/OBAR
29     K18 = K*OBAR/4.
30     T = (ALOG(O180) - ALOG(O181))/K18
31     C
32     C      CALCULATE TRANSITION PROBABILITIES
33     C
34     P11 = (1.-P)*(1. + 3.*P/(4.-3.*P))
35     P10 = 1.-P11
36     P22 = (1. - P)*(1. + P/(2. - P))
37     P21 = (1.-P22)*P11
38     P20 = (1.-P22)*P10
39     P33 = (1.-P)*(1.+P/(4.-P))
40     P32 = (1.-P33)*P22
41     P31 = (1.-P33)*P21
42     P30 = (1.-P33)*P20
43     P44 = 1.-P
44     P43 = (1.-P44)*P33
45     P42 = (1.-P44)*P32
46     P41 = (1.-P44)*P31
47     P40 = (1.-P44)*P30
48     S4 = P43 + P42 + P41 + P40
49     S3 = P32 + P31 + P30
50     S2 = P21 + P20
51     S1 = P10
52     C
53     C      CALCULATE DISTRIBUTION FOR FINAL % 180
54     C
55     H4 = H40*EXP(-K*S4*T)
56     C43 = P43*H40/(S3-S4)
57     H3 = (H30-C43)*EXP(-K*S3*T) + C43*EXP(-K*S4*T)
58     C42 = (P42*H40 + P32*C43)/(S2-S4)
59     C32 = P32*(H30-C43)/(S2-S3)
60     H2 = (H20 - C42 - C32)*EXP(-K*S2*T) + C42*EXP(-K*S4*T)

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61      H1 = C32*EXP(-K*S3*T)
62      C41 = (P41*H40 + P31*C43 + P21*C42)/(S1-S4)
63      C31 = (P31*(H30-C43) + P21*C32)/(S1-S3)
64      C21 = P21*(H20 - C42 - C32)/(S1 - S2)
65      H1 = (H10 - C41 - C31 - C21)*EXP(-K*S1*T) +
66      1 C41*EXP(-K*S4*T) + C31*EXP(-K*S3*T) + C21*EXP(-K*S2*T)
67      C40 = (P40*H40 + P30*C43 + P20*C42 + P10*C41)/(S4)
68      C30 = (P30*(H30 - C43) + P20*C32 + P10*C31)/S3
69      C20 = (P20*(H20-C42-C32) + P10*C21)/S2
70      C10 = (P10*(H10-C41-C31-C21))/S1
71      HO = C40*(1.-EXP(-K*S4*T)) + C30*(1.-EXP(-K*S3*T)) +
72      1 C20*(1.-EXP(-K*S2*T)) + C10*(1.-EXP(-K*S1*T)) + H00
73      C
74      C      CALCULATE % 18 O FROM H VALUES AS CHECK
75      C
76      OTOTAL = HO + H1 + H2 + H3 + H4
77      O18 = .25*H1 + .5*H2 + .75*H3 +H4
78      ENRICH = (O18/OTOTAL) * 100.
79      C
80      C      OUTPUT
81      C
82      WRITE(6,15)
83      WRITE(6,10) P,X,OBAR,OP,R
84      WRITE(6,50)
85      WRITE(6,60) S4,P40,P41,P42,P43,P44
86      WRITE(6,60) S3,P30,P31,P32,P33
87      WRITE(6,60) S2,P20,P21,P22
88      WRITE(6,60) S1,P10,P11
89      WRITE(6,61)
90      WRITE(6,13)
91      WRITE(6,11) TO,O18O,H0O,H1O,H2O,H3O,H4O
92      WRITE(6,11) T, ENRICH, HO,H1,H2,H3,H4
93      101 CONTINUE
94      200 CONTINUE
95      7 FORMAT(5F10.4)
96      8 FORMAT(2F10.4)
97      10 FORMAT (1H0,5(E14.7,5X),///)
98      11 FORMAT (1H ,F5.2,6X,F6.2,10X,5(F7.1,4X),/)
99      13 FORMAT (6H TIME,6X,7H % O18,9X,5H HO,6X,5H H1,6X,
100      1 5H H2,6X,5H H3,6X,5H H4,/)
101      15 FORMAT (5H1 PC,15X,8H K2/K-1,11X,7H OBAR,12X,
102      1 10H O PRIME,9X,6H R4,/)
103      50 FORMAT (6H S,10X,6H PO,9X,6H P1,9X,
104      1 6H P2,9X,6H P3,9X,6H P4,/)
105      60 FORMAT (1H ,6(F10.6,5X))
106      61 FORMAT (////)
107      STOP
108      END
END OF FILE

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XI. Appendix 2

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Biochemistry

Capacity for alternating sites cooperativity in catalysis by succinyl-coenzyme A synthetase

(enzyme subunits/phosphoenzyme/phosphohistidine)

WILLIAM T. WOŁODKO, MAUREEN D. O'CONNOR, AND WILLIAM A. BRIDGER

Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada, T6G 2H7

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ABSTRACT Succinyl-coenzyme A synthetase [succinate:CoA ligase (ADP-forming), EC 6.2.1.5] of *Escherichia coli* is an $\alpha_2\beta_2$ tetramer. A histidyl residue in the α subunit is phosphorylated as a catalytic intermediate. It has been suggested [Bild, G. S., Jansson, C., & Boyer, P. D. (1980) *J. Biol. Chem.* 255, 8109-8115] that the mechanism of action of this enzyme involves intersubunit cooperativity in which attachment of substrates at one of the two active sites promotes catalytic events at the other. This scheme would require that the two active sites, although otherwise equivalent, should act alternately.

We have prepared a hybrid enzyme species that contains one ^{35}S -labeled α subunit (dephosphorylated), one nonradioactive α subunit (phosphorylated), and two β subunits per tetrameric molecule. With the aid of a selective chromatographic procedure for the isolation of peptides that contain phosphohistidyl residues, we have shown that each of the α subunits undergoes phosphorylation when the hybrid enzyme is exposed briefly to substrates. This result demonstrates that the two active sites are capable of alternate activity and lends support to the concept of alternating sites cooperativity. The half-of-the-sites phosphorylation that occurs with this enzyme is not a consequence of permanent asymmetry or other lack of equivalence of the two α subunits.

Most enzymes are composed of subunits. Rationales for oligomeric structures are often clearly evident. These include homotropic cooperativity and related allosteric phenomena (e.g., ref. 1); the possibility for shared active sites between subunits, as exemplified by phosphofructokinase (2) and other enzymes; and the capacity for more effective folding or enhanced stability in a multienzyme structure (3).

Succinyl-CoA synthetase [succinate: CoA ligase (ADP-forming), EC 6.2.1.5] of *Escherichia coli* has an $\alpha_2\beta_2$ subunit structure (4). It now seems certain that the reason for two kinds of subunits in the enzyme is that the active site overlaps both α and β . Whereas the β subunit contains sites for attachment of the substrate succinate and CoA (5, 6), the α subunit binds ATP and contains the active-site histidine residue that is phosphorylated as a catalytic intermediate (7-9). Less clear, however, is the rationale for the presence of two copies of each subunit in the tetrameric enzyme. The results that we report here demonstrate the capacity for alternate activity of the two catalytic centers of the $\alpha_2\beta_2$ tetramer. This ability is essential to the concept of alternating sites cooperativity that has been proposed for this enzyme; this concept generally visualizes attachment of substrates at one active site promoting catalytic events at the other (8).

MATERIALS AND METHODS

Preparation of Succinyl-CoA Synthetase and Its Subunits. Cultures of *E. coli* (Crooks strain) were grown on a phosphate-

buffered, succinate-based medium and treated as described (9). Bacteria to be labeled with the radioisotope ^{35}S were grown under similar conditions, except that the total volume was 10 liters. Carrier-free $\text{H}_2^{35}\text{SO}_4$ (10 mCi; 1 Ci = 3.7×10^{10} becquerels; New England Nuclear, Canada) was mixed with the metal/salt solution, and the mixture was sterilized by membrane filtration prior to addition to the bulk of the sterile growth medium. Succinyl-CoA synthetase was purified essentially by the procedure of Leitzmann *et al.* (10), with addition of a final purification step involving affinity chromatography on Blue Sepharose CL-6B. The protein concentration of the purified enzyme was measured by absorbance at 280 nm, with a value of 5.0 for ϵ (11). Succinyl-CoA synthetase was dissociated into its subunits by treatment with an acidic urea solution, and the individual subunits were subsequently isolated by gel filtration as described (12). The subunits were stored at 4°C in a solution containing 6 M urea, 5% (vol/vol) acetic acid, 0.5 mM dithiothreitol, and 0.1 mM EDTA. The protein concentrations of the purified α and β subunits were determined spectrophotometrically at 280 nm, with values of ϵ equal to 2.75 and 6.8, respectively (unpublished data).

Preparation of the Phosphorylated α Subunit. Purified α subunit was phosphorylated as described (5). An aliquot of a stock α -subunit solution (containing approximately 3 mg of protein) was diluted with 1 mM ATP/1 mM dithiothreitol/10 mM MgCl_2 /0.1 M Tris-HCl, pH 8.3, to final protein and urea concentrations of 0.04-0.05 mg/ml and 0.2-0.3 M, respectively. The final pH was 7.4-7.5 (adjusted with HCl, if necessary). This mixture was incubated at 25°C for a minimum time of 40 hr, then cooled to 4°C and concentrated with a Millipore Immersible Concentrator (nominal molecular weight cutoff of 10,000). Excess ATP was removed from the phosphorylated subunit preparation by gel exclusion chromatography on a 1.0×50 cm column of Sephadex G-25 (fine) equilibrated and eluted at 4°C with 0.1 mM EDTA/0.5 mM dithiothreitol/50 mM Tris-HCl, pH 7.4.

Preparation of Hybrid Enzyme. The recombination of ^{35}S -labeled α subunit, phosphorylated α subunit, and β subunits into active enzyme was carried out by fulfilling the following conditions. The ratio of ^{35}S -labeled α subunit to phosphorylated α subunit to β subunits in the refolding mixture was 1:1:2 on a molar basis. The "refolding buffer" consisted of 10 mM MgCl_2 , 1 mM dithiothreitol, and 50 mM Tris-HCl, (pH 7.4), and the volume used brought the final concentrations of the protein and urea in the refolding mixture to 0.12 mg/ml and <0.4 M, respectively.

A typical protocol called for addition of stock solutions of purified ^{35}S -labeled α subunit and β subunits into a beaker containing an equal volume of 1 M Tris (to neutralize the acetic acid). This mixture was diluted immediately with the appropriate volume of refolding buffer; finally, the phosphorylated α subunit stock solution was added. The final pH was equal to 7.3-7.4. The refolding mixture was incubated at 25°C for 180

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min, then cooled to 4°C, and concentrated to approximately 3 ml with a Millipore Immersible Concentrator. The hybrid enzyme was purified by gel exclusion chromatography on a 1.5 × 60 cm column of Sephacryl 200 (superfine) equilibrated and eluted with 0.1 mM EDTA/70 mM Tris-HCl, pH 7.2.

Enzyme Assay. Succinyl-CoA synthetase activity was measured by the direct spectrophotometric method (13) based on the increase in absorbance at 232 nm that accompanies thioester formation. A unit of activity is defined as that catalyzing the formation of 1 μmol of succinyl-CoA per min at 25°C.

Preparation of ³²P-Labeled Succinyl-CoA Synthetase. Native enzyme was phosphorylated with ³²P for use as a tracer in chromatographic separations (see below). The reaction mixture consisted of 1 mg of purified succinyl-CoA synthetase and 75 pmol of [γ-³²P]ATP (0.3 Ci/μmol; New England Nuclear, Canada) in 1 ml of 10 mM MgCl₂/50 mM Tris-HCl, pH 7.2. The reaction was started by the addition of the ATP, carried out at 22°C for 60 sec, and quenched with the addition of 0.5 ml of 0.35 M EDTA/50 mM Tris-HCl, pH 8.2. The quenched mixture was desalted, and unreacted [³²P]ATP was removed by chromatography on a 0.7 × 13.5 cm column of Sephadex G-25 (fine) topped with a 0.7 × 1 cm layer of Bio-Rad AG1-8X ion-exchange resin. This column was equilibrated and eluted with 50 mM KCl/50 mM Tris-HCl, pH 8.1.

Procedure for Tryptic Digestion. Protein to be subjected to digestion with trypsin was first treated with a solution containing 2 M urea for 1 hr at 37°C; such treatment was found to loosen the structure of succinyl-CoA synthetase and to facilitate subsequent proteolysis. The incubation mixture consisted of 2 M urea, 5 mM CaCl₂, 35 mM KCl, and 50 mM Tris-HCl (pH 8.1). Tracer succinyl-CoA synthetase labeled with ³²P was treated in an identical manner. Proteolysis was carried out by the sequential addition of solid bovine pancreatic trypsin [281 units/mg, treated with *N*-tosyl-L-phenylalanine chloromethyl ketone (Worthington)]. Approximately 0.4 mg of trypsin was added to the mixture of protein and succinyl-CoA synthetase-tracer immediately after the treatment with urea and twice more at 2-hr intervals thereafter. The tryptic digestion was conducted at 37°C for a total of 5 hr. The digest was subsequently cooled to 20°C and subjected to ion-exchange chromatography.

Column Chromatography. Ion-exchange chromatography was carried out at 21°C on 0.7 × 29 cm columns of QAE-Sephadex A-25 equilibrated with 2 M urea/50 mM KCl/50 mM Tris-HCl, pH 8.1. The standard elution protocol was as follows: application of sample, elution with the equilibration buffer (1.5 × the column volume), application of a 36-ml linear gradient with respect to KCl (50–300 mM KCl in the same buffer), and final washing with 30 ml of 300 mM KCl in the same buffer. Material that was pooled from the foregoing fractionation of tryptic digests was desalted by gel exclusion chromatography on a 1 × 32 cm column of Sephadex G-10 equilibrated and eluted with 0.01 M NH₄OH at 4°C.

Measurement of Radioactivity. Radioactivity was measured using Aquasol (New England Nuclear) with a Beckman LS-230 liquid scintillation counter equipped with fixed-window isotope modules.

Gel Electrophoresis. Disc gel electrophoresis on 7% (wt/vol) polyacrylamide gels was carried out as described (14).

RESULTS

Experimental Plan. Catalysis by succinyl-CoA synthetase occurs with the intermediate participation of a phosphoenzyme, E-P, containing a phosphohistidine residue in the α subunit (4) as shown in the following series of reactions:

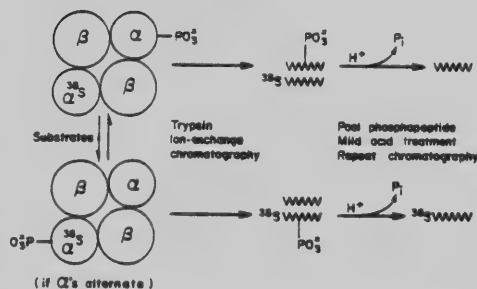
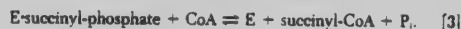


FIG. 1. Design of experiment to test the capacity of the α subunits for alternate activity.



Formation of E-P provides a convenient method for labeling a functional active site. Repeated measurements in this laboratory (15) have shown that succinyl-CoA synthetase exhibits half-of-the-sites reactivity with respect to E-P formation; only one phosphoryl group is incorporated per α₂β₂ tetramer after treatment with excess ATP. The question addressed here is whether this behavior is caused by (i) preexisting asymmetry with one α subunit predestined to be inactive or (ii) alternating activity of the two α subunits in a situation in which constraints of the mechanism preclude simultaneous phosphorylation of the two sites. The latter explanation, as discussed below, is in keeping with the concept of alternating sites cooperativity.

To test the capacity for alternate activity of the two sites, we developed the experimental scheme outlined in Fig. 1. The plan involved preparation of a hybrid enzyme containing ³⁵S-label in one of the two α subunits and phosphohistidine in the other. After the putative alternating activity, the location of the phosphoryl group should be scrambled to produce a species in which the ³⁵S-labeled subunit is also phosphorylated. Because we have been unable to develop reliable methods for the separation of α and α-P subunits, the scheme included exploiting a method that we have devised for the isolation of peptides containing phosphohistidine (16). In this method, a trypsin digest is subjected to ion-exchange chromatography, the phosphopeptide fraction is treated with dilute acid to remove the phosphoryl group by hydrolysis, and repeat chromatography under the same conditions results in specific early elution of the previously phosphorylated peptide by virtue of its loss of net negative charge. Because the phosphohistidine-containing peptide contains NH₂-terminal methionine (16), the peptide thus isolated should contain ³⁵S, provided that scrambling of the phosphoryl group has occurred.

Preparation of Hybrid Enzyme. In accordance with the foregoing scheme, we prepared hybrid enzyme by refolding a mixture of individual subunits to produce tetramer containing two β subunits, one ³⁵S-labeled α subunit (unphosphorylated), and one unlabeled α-P-subunit. The refolded mixture was purified by gel filtration, effectively separating active refolded enzyme from inactive aggregated material (Fig. 2A and B). The specific catalytic activity of the hybrid enzyme (≈ 48 units/mg through the peak) was equivalent to that expected for pure succinyl-CoA synthetase.* Fig. 2C shows that the active fractions contained

* The presence of inactive material in Fig. 2A may explain why previously reported reconstitution attempts without subsequent gel filtration yielded only 50–60% of the theoretical specific activity (12).

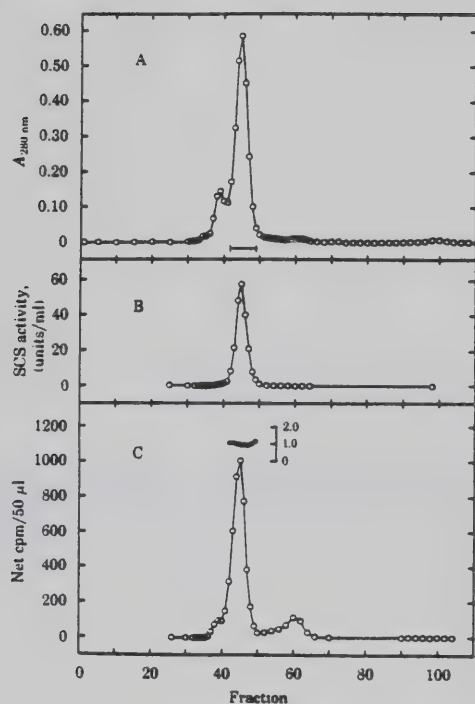


FIG. 2. Purification of hybrid enzyme by gel exclusion chromatography. The sample applied to the column was obtained by refolding a mixture of ^{35}S -labeled α subunits (dephosphorylated), nonradioactive phosphorylated α subunits, and unlabeled β subunits. (A) Absorbance profile. The bar indicates the fractions that were pooled to make up the stock hybrid enzyme solution used in subsequent experiments. (B) Profile of succinyl-CoA synthetase (SCS) activity. (C) Distribution of ^{35}S . (Inset) Molar ratio of ^{35}S -labeled α subunit per enzyme tetramer, calculated from the specific radioactivity of the pure ^{35}S -labeled α subunit.

one ^{35}S -labeled α subunit per tetramer, as expected. The equivalence of the hybrid enzyme to the native species was confirmed by gel electrophoresis (Fig. 3); the purified hybrid (lane B) migrated identically to native E-P (lane C) and contained no detectable free α or β subunits (lanes D and E, respectively). Presence of significant amounts of dephosphorylated tetramer is ruled out by the specific activity of the hybrid enzyme at the theoretical limit [dephosphorylated refolded enzyme is inactive (12)] and by the homogeneity of both protein stain and radioactivity in the electrophoretic pattern. Therefore, the reassembly of the hybrid enzyme has not followed a binomial distribution but has strongly favored the desired monophosphorylated tetramer.

Test for Alternating Sites. This experiment was performed as outlined in Fig. 1. A portion of the hybrid enzyme ($[\text{P}^{35}\text{S}]\alpha_1\alpha_2\text{P}_1\beta_2$) was exposed briefly to unlabeled substrates, sufficient to allow for approximately 150 turnovers. An equivalent sample of hybrid enzyme that had not been treated with substrates was used as a control. To each sample was then added a small amount of ^{125}P -labeled E-P to serve as a tracer of phosphopeptide. The samples were then treated in identical fashion with trypsin, and

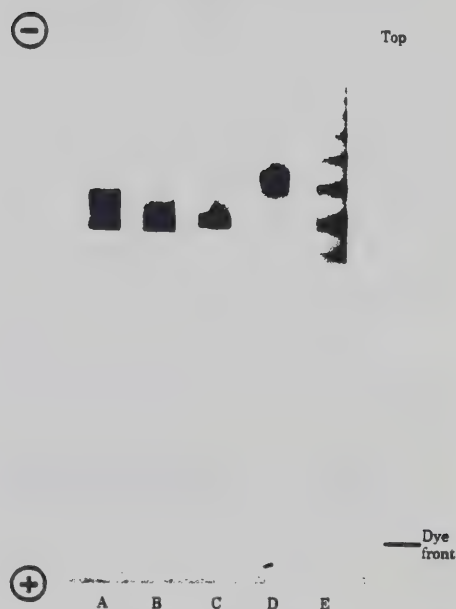


FIG. 3. Polyacrylamide gel electrophoresis analysis of the purity of the hybrid enzyme. Electrophoresis was carried out in 7% gels (non-dissociating conditions). Lanes: A, stock α and β subunits refolded under the same conditions used for the preparation of the hybrid enzyme but without purification by gel filtration (sample = 18 μg of protein); B, hybrid enzyme (18 μg) purified by gel filtration (see Fig. 2); C, phosphorylated succinyl-CoA synthetase (10 μg); D, purified α subunit (12 μg); E, purified β subunit (18 μg) which aggregates on the gel.

the digests were subjected to anion-exchange chromatography on QAE-Sephadex A-25. Fig. 4 shows the resulting chromatograph for the sample that had been exposed to substrates: the control was virtually identical. Guided by the ^{32}P -labeled tracer, we pooled fractions containing phosphopeptide from both digests, and these were desalted on Sephadex G-10. The solutions were then adjusted to pH 2.0 by addition of concentrated HCl and incubated at 37°C for 12 hr to allow for hydrolysis of the NP bond of the phosphohistidyl residue. The acid digests were concentrated by lyophilization and reapplied to identical QAE-Sephadex A-25 columns. The results show clearly that the mild acid hydrolysis gave rise to an ^{35}S -labeled active-site peptide in the chromatographic breakthrough (Fig. 5A), whereas there was no significant ^{35}S -labeled material produced in the control experiment (Fig. 5B). The recovery of ^{35}S -labeled peptide was not trivial, because knowing the specific radioactivity of the ^{35}S -labeled hybrid, we calculated that fractions 6–8 of Fig. 5A contained approximately 0.25 mol of ^{35}S -labeled peptide per mole of hybrid enzyme that was used to begin the experiment. (The theoretical limit is 0.5, if one assumes complete recovery at each stage and complete scrambling of phosphoryl group between labeled and unlabeled α -subunits.)

DISCUSSION

Alternating sites cooperativity describes a situation in which equivalent active sites participate in catalysis sequentially, with

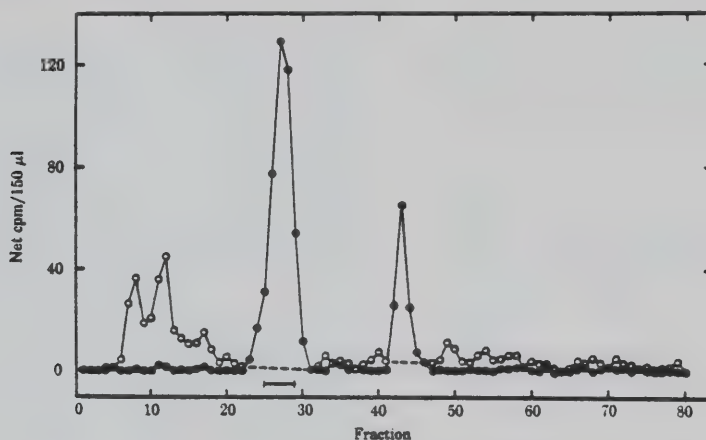


FIG. 4. Anion-exchange chromatographic fractionation of the tryptic digest of the mixture of hybrid enzyme and ^{32}P -labeled tracer enzyme on QAE-Sephadex A-25. Fraction vol = 1.1 ml. \circ , ^{35}S profile from hybrid enzyme; \bullet , ^{32}P profile from tracer enzyme. Fractions 25–29 represent the phosphohistidine-containing peak and were pooled for the next step of the experiment. ---, Regions where ^{35}S radioactivity could not be reliably measured. The results shown are for the hybrid enzyme sample that had been exposed to substrates; the profile for the control sample was indistinguishable.

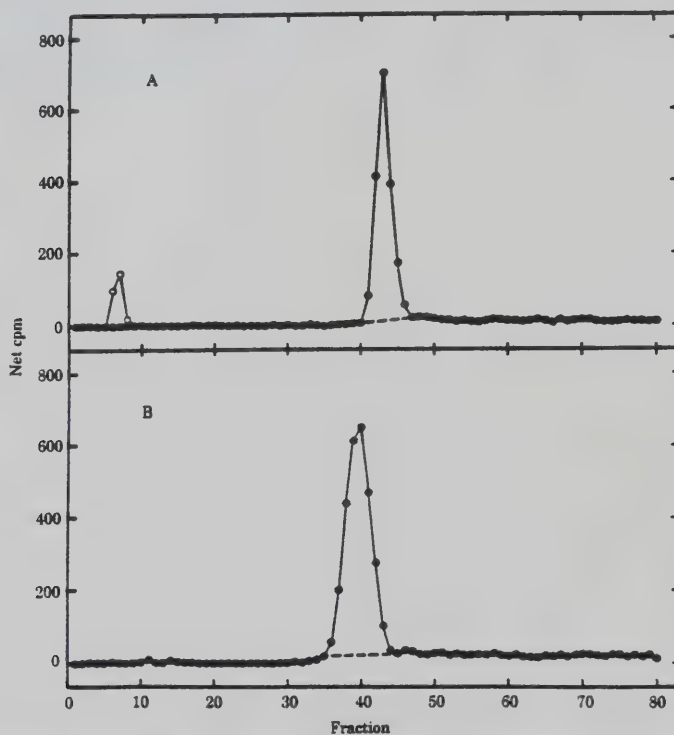


FIG. 5. Repeat anion-exchange fractionation of the pooled phosphopeptide fractions from Fig. 4 after mild acid hydrolysis. Fraction vol = 1.1 ml. Radioactivity was measured by counting the entire fraction in 10 ml of Aquasol. \circ , ^{35}S radioactivity; \bullet , ^{32}P radioactivity. A, Experiment in which the hybrid enzyme was exposed to substrates. B, Control experiment without exposure of hybrid enzyme to substrates.

binding of substrate at one site promoting catalytic events at another. This concept provides a rationale that may contribute to the prevalence of multisubunit structures in enzymology. In the case of *E. coli* succinyl-CoA synthetase, Bild *et al.* (8) have reported that ^{18}O -labeled P/succinate exchange is modulated by the concentration of ATP in a manner consistent with alternating sites cooperativity. These workers have suggested that binding of ATP to, or phosphorylation of, one active site may promote a conformational change at the other site, resulting in either facilitated release of product succinyl-CoA or enhanced rates of certain intermediate steps. Clearly, any such model for subunit cooperation during catalysis requires that the individual sites are capable of alternate activity.

The scrambling of the phosphoryl group between unlabeled and ^{35}S -labeled α subunits that we demonstrate here establishes that both active sites are available and capable of catalytic activity. Although this experiment does not prove that the subunits are required to function in strictly alternating sequence, the scrambling is entirely compatible with this concept. Obviously, we do not have a situation where one-half of the enzyme contains the only functional active site, with the other potentially active site buried or nonfunctional because of fixed asymmetry.

Finally, recent experiments in this laboratory (unpublished data) have shown that the relative rate of the ^{18}O /phosphate/succinate exchange is modulated not only by ATP, as reported by Bild *et al.* (8), but also by enzyme concentration, with the exchange rate substantially diminished at high dilutions. These observations raise the possibility that the proposed ATP-driven intersite cooperativity may involve enzyme dissociation and reassociation.

We thank Edward R. Brownie for his skilled assistance in the preparation of both radioactive and unlabeled succinyl-CoA synthetase and its subunits. M.D.O.C. was the recipient of a Medical Research Council Studentship. This research was supported by Grant MT-2805 from the Medical Research Council of Canada.

1. Fletterick, R. J. & Madsen, N. B. (1990) *Annu. Rev. Biochem.* **49**, 31-61.
2. Evans, P. R. & Hudson, P. J. (1979) *Nature (London)* **279**, 500-504.
3. Chan, W. W. C. (1976) *Can. J. Biochem.* **54**, 521-528.
4. Bridger, W. A. (1974) in *The Enzymes*, ed. Bover, P. D. (Academic, New York), 3rd Ed., Vol. 10, pp. 581-606.
5. Pearson, P. H. & Bridger, W. A. (1975) *J. Biol. Chem.* **250**, 8524-8529.
6. Collier, G. E. & Nishimura, J. S. (1978) *J. Biol. Chem.* **253**, 4938-4943.
7. Bridger, W. A. (1971) *Biochem. Biophys. Res. Commun.* **42**, 948-954.
8. Bild, G. S., Janson, C. A. & Boyer, P. D. (1980) *J. Biol. Chem.* **255**, 8109-8115.
9. Wolodko, W. T., Brownie, E. R. & Bridger, W. A. (1980) *J. Bacteriol.* **143**, 231-237.
10. Leitzmann, C., Wu, J.-Y. & Boyer, P. D. (1970) *Biochemistry* **9**, 2338-2346.
11. Krebs, A. & Bridger, W. A. (1974) *Can. J. Biochem.* **53**, 594-598.
12. Pearson, P. H. & Bridger, W. A. (1975) *J. Biol. Chem.* **250**, 4451-4455.
13. Bridger, W. A., Ramaley, R. F. & Boyer, P. D. (1969) *Methods Enzymol.* **13**, 70-75.
14. Gabriel, O. (1971) *Methods Enzymol.* **22**, 565-578.
15. Moffet, F. J., Wang, T.-T. & Bridger, W. A. (1972) *J. Biol. Chem.* **247**, 8139-8144.
16. Wang, T., Jurasek, L. & Bridger, W. A. (1972) *Biochemistry* **11**, 2067-2070.

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